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Award Number: DAMD17-97-1-7228

TITLE: Growth Receptors and Integrins in Breast Cancer

PRINCIPAL INVESTIGATOR: Yoji Shimizu, Ph.D.

CONTRACTING ORGANIZATION: University of Minnesota
Minneapolis, Minnesota 55415-1226

REPORT DATE: November 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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20001107 055

REPORT DOCUMENTATION PAGE*Form Approved*
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)**2. REPORT DATE**
November 1999**3. REPORT TYPE AND DATES COVERED**
Final (1 Sep 97 - 31 Aug 99)**4. TITLE AND SUBTITLE**

Growth Receptors and Integrins in Breast Cancer

5. FUNDING NUMBERS

DAMD17-97-1-7228

6. AUTHOR(S)

Yoji Shimizu, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Minnesota

Minneapolis, Minnesota 55415-1226

**8. PERFORMING ORGANIZATION
REPORT NUMBER****E-MAIL:**

shimi002@gold.tc.umn.edu

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES****12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

The central hypothesis of this IDEA Award is that epidermal growth factor (EGF) receptors in breast cancer transduce intracellular signals that lead to activation of the lipid kinase phosphoinositide 3-OH kinase (PI 3-K) and rapid increases in the functional activity of integrin adhesion receptors, thereby regulating breast cancer cell motility and invasiveness. During the course of this IDEA Award, we have established the validity of this hypothesis. EGF and betacellulin, ligands that activate the EGFR, and heregulin- β , a ligand that activates erbB3 and erbB4, induce dose- and time-dependent adhesion of MDA-MB-435 cells to type IV collagen via β 1 integrins. These same growth factors also upregulate β 1 integrin-mediated adhesion on collagen and laminin. Inhibition of PI 3-K activity blocks both adhesion and migration induced by these growth factors. Antibody blocking studies further suggest differential recruitment of erbB2 to the erbB3 receptor rather than to the EGFR upon growth factor binding to MDA-MB-435 cells. In other breast carcinoma cell lines, there are intriguing differences in the requirement for PI 3-K in EGF versus heregulin- β -mediated adhesion pathways. Thus, EGFR family members regulate β 1 integrin function on breast cancer cells via PI 3-K activation.

14. SUBJECT TERMS

Breast Cancer

15. NUMBER OF PAGES

54

16. PRICE CODE**17. SECURITY CLASSIFICATION
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION
OF ABSTRACT**

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

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X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.


PI Signature

11-26-99
Date

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5. INTRODUCTION

The adhesive behavior of tumor cells is dictated by the spectrum of adhesion receptors expressed on the cell surface. Consequently, quantitative changes in the expression of the integrin family of adhesion receptors result in dramatic effects on tumor growth and metastasis. However, the issue of qualitative, rather than quantitative, regulation of integrins and its functional relevance to breast cancer has not been addressed. The central focus of this IDEA Award has been to test the hypothesis that an unappreciated but critical role for growth factor receptors in breast cancer is to transduce intracellular signals that rapidly increase the functional activity of integrin adhesion receptors, thereby regulating breast cancer cell motility and invasiveness. Specifically, we hypothesized that: 1) exposure of breast carcinoma cells to epidermal growth factor (EGF) results in the generation of an intracellular signaling cascade that results within minutes in the rapid upregulation of the functional activity of $\beta 1$ integrin adhesion receptors; 2) EGF-dependent activation of integrin function is dependent on EGF receptor (EGFR)-mediated activation of the lipid kinase phosphatidylinositol 3-kinase (PI 3-K); and 3) inhibition of EGF-mediated activation of PI 3-K will block breast cancer cell motility and invasiveness induced by EGF-dependent activation of $\beta 1$ integrin functional activity. This model of growth factor receptor regulation of breast cancer cell adhesion was addressed in two specific aims. First, various breast cancer cell lines were analyzed for EGF-mediated activation of PI 3-K, increases in integrin-mediated adhesion to various extracellular matrix (ECM) ligands (fibronectin, type I collagen, type IV collagen, laminin), and integrin-dependent cell motility and cell invasion. Second, a role for PI 3-K in EGF-mediated regulation of integrin adhesiveness, cell motility and cell invasion was addressed by determining if these EGF-dependent responses could be blocked by pharmacological or genetic inhibitors of PI 3-K.

6. BODY

6.A. EXPERIMENTAL METHODS. All experimental methods are described in detail in the appended publication by Adelsman et al. entitled "Stimulation of $\beta 1$ -integrin function by epidermal growth factor and heregulin- β has distinct requirements for erbB2 but a similar dependence on phosphoinositide 3-OH kinase". This manuscript was published in the September 1999 issue of *Molecular Biology of the Cell* [1] and is enclosed in the appendix of this final report.

6.B. RESULTS AND DISCUSSION

Adhesion of breast carcinoma cell lines to $\beta 1$ integrin ligands can be regulated by stimulation of EGFR family members. In order to test our hypothesis that EGF stimulation might upregulate $\beta 1$ integrin function in breast cancer cells, we examined a variety of breast carcinoma cell lines, both metastatic and non-metastatic in nature, for their ability to respond to EGF stimulation by upregulating adhesion to $\beta 1$ integrin extracellular matrix (ECM) ligands. Several cell lines were examined, including the highly metastatic lines MDA-MB-435 [2], MDA-MB-361, and SKBR3, and the less metastatic cell lines MDA-MB-231 [2,3], T47D, MCF7 [3,4], and A431. The cell lines responded in a heterogeneous fashion with regard to adhesion to matrix ligands such as FN, Type IV collagen (COLL), and laminin (LAM) or merosin (MER). For example, EGF treatment increased adhesion of MDA-MB-435 cells to FN, COLL, MER, and LAM (Figure 1A) and [1]. However, in line with previous observations [5], low doses of EGF had little effect on A431 cell adhesion and a negative effect at higher doses of EGF for adhesion of A431 cells to FN (Figure 1B) or COLL (Figure 1C). While the A431 cell line dramatically overexpresses the EGFR, other cell lines with high levels of EGFR, such as SKBR3 and MDA-

MB-231, were not inhibited in adhesion by stimulation with EGF in our assays, further underscoring the complexity of these regulatory pathways in a given defined cell system. Indeed, the SKBR3 cells showed significantly increased adhesion to FN and COLL and a smaller increase on MER in response to EGF (Figure 1D), while MDA-MB-231 cells routinely showed little stimulated adhesion to FN or COLL (Figure 1E). However, the MDA-MB-435, T47D (Figure 1F) and SKBR3 cells routinely showed increased adhesion to these ECM components in response to EGF stimulation. However, all of the cell lines showed increased adhesion in response to the activating $\beta 1$ integrin-specific mAb TS2/16 [6,7], which directly binds to the $\beta 1$ integrin subunit and induces a conformational change that increases adhesion (Figure 1). This suggests that the heterogeneous response of these cell lines to EGF is due primarily to differences in EGFR signaling, rather than inherent differences in $\beta 1$ integrin function. Because of the inherent complexity involved in studying multiple cell lines, and due to the observation of strong $\beta 1$ integrin-dependent adhesion on collagen for the MDA-MB-435 cells, the majority of the subsequent studies utilized the MDA-MB-435 cell system.

Adhesion of MDA-MB-435 cells to type IV collagen was specifically induced by exposure to the growth factor EGF, although to less extent than by directly activating the $\beta 1$ integrin with TS2/16 [6,7]. $\beta 1$ integrins were the major adhesion receptors responsible for this event as indicated by the nearly complete inhibition of both unstimulated and stimulated (TS2/16 or EGF) adhesion to collagen by these cells by inhibitory $\beta 1$ integrin-specific antibodies [1]. Adhesion to FN and MER was typically blocked less effectively with inhibitory $\beta 1$ integrin-specific antibodies, leaving open the possibility of contributions by other adhesion receptors. The EGF-mediated adhesion to collagen was dose dependent, with maximal stimulation of adhesion peaking at 10 ng/ml EGF with a plateau of maximal adhesion at higher concentrations of growth factor [1]. Based on these early findings, we typically stimulated cells with 100 ng/ml EGF in cellular adhesion assays. The EGF-stimulated adhesion to collagen was also induced in a rapid fashion, with maximal increases over unstimulated adhesion found by 10-20 minutes of stimulation at 37°C [1].

MDA-MB-435 cells express moderate levels of the EGFR, erbB2, and erbB3, with no detectable erbB4 protein by flow cytometry or blotting methods [1]. Since erbB2 is still considered an "orphan" receptor in that no growth factor has yet been found by which erbB2 is directly bound and activated, the presence of both EGFR and erbB3 on the surface of these cells led us to investigate additional growth factors that bind to and activate either the EGFR or erbB3. Betacellulin stimulation of the EGFR also increased MDA-MB-435 cell adhesion to collagen in a dose-dependent fashion, with the maximal stimulation of adhesion paralleling that found with EGF stimulation in the same assay [1]. Examination of heregulin- α (HRG α), a growth factor that binds only to erbB3 or erbB4, showed no effects on adhesion of this cell line at any of the concentrations of growth factor tested [1]. In contrast, the more recently described heregulin isoform, heregulin- β (HRG β), showed a striking increase in adhesion of these cells to collagen with increasing levels of growth factor [1]. The stimulatory effect of HRG β was slightly less than direct activation of $\beta 1$ through the TS2/16 antibody, and was routinely higher than EGF stimulation. These data suggested that stimulation through either the EGFR with ligands such as EGF or betacellulin, or stimulation of the erbB3 receptor with HRG β , could significantly stimulate $\beta 1$ integrin-dependent adhesion of the MDA-MB-435 cell line to type IV collagen. Importantly, this upregulation occurred in the absence of significant changes in either integrin subunit or EGFR family receptor numbers on the cell surface as assessed by flow cytometry [1].

Stimulation of EGFR family members increases migration of MDA-MB-435 breast carcinoma cells. As the MDA-MB-435 cells are highly metastatic in nude mouse models [2], we also examined cellular migration and motility *in vitro*. Previous studies described migration and adhesion of unstimulated MDA-MB-435 cells on laminin and collagen [8], and our FACS analyses shows strong expression of $\beta 1$ integrins capable of binding collagen and laminin ($\alpha 2$, $\alpha 3$, $\alpha 6$; data not shown), consistent with this earlier study [8]. Additionally, both collagen and laminin represent critical physiological components for cellular metastatic motility [9-11]. Thus, we examined the migration of unstimulated and stimulated MDA-MB-435 cells on both collagen and laminin. As found in our adhesion experiments, betacellulin, EGF, and HRG β all increased the migration of 435 cells toward laminin [1] in a dose-dependent fashion, while parallel assays showed similar results on collagen (data not shown). While the adhesion experiments showed increasing growth factor-stimulated adhesion with a plateau response [1], betacellulin and EGF both demonstrated bell-shaped curves for stimulated migration, with maximal responses at 0.1-1 ng/ml and 1-10 ng/ml for betacellulin and EGF, respectively. Migration in response to HRG β showed similar dose-effects as seen for adhesion with strong induction of migration, reaching a maximal response by 100-250 ng/ml growth factor. While the stimulated adhesion by HRG β was consistently higher than that mediated by EGF, both events were $\beta 1$ integrin-dependent as illustrated by the ability of an inhibitory $\beta 1$ integrin-specific antibody to completely abrogate both unstimulated and EGF- or HRG β -stimulated migration towards collagen or laminin [1]. Thus, our adhesion and migration data support the hypothesis that growth factor stimulation of the EGFR couples to $\beta 1$ integrin-mediated functional events. Further, we have found that erbB3, another receptor in the EGFR family, mediates stimulation of both adhesion and cell migration by the growth factor HRG β .

Contribution of dimerization partners with the EGFR and erbB3 in EGF and HRG β regulation of $\beta 1$ integrins. The observation that two mechanistically distinct growth factors, EGF and HRG β , were capable of stimulating $\beta 1$ integrin activity, coupled with the complex heterodimerization potential of the EGFR family of receptors [12,13], led us to investigate the potential contribution of dimerization partners with the EGFR or with erbB3 in mediating the effects of EGF and HRG β on $\beta 1$ integrin function. An anti-erbB3 antibody that blocks HRG β binding [14] specifically abrogated HRG β -induced adhesion of MDA-MB-435 cells without affecting EGF or TS2/16 stimulated adhesion, even at high concentrations of antibody [1]. Additionally, an anti-erbB2 antibody that blocks the effects of EGF or HRG β binding to the dimerization partners of erbB2 [15] negated HRG β -stimulated adhesion without specifically affecting EGF or TS2/16 stimulation conditions [1]. The combination of both anti-erbB3 and anti-erbB2 antibodies gave a slightly stronger reduction in adhesion, further supporting the contribution of both erbB2 and erbB3 in HRG β -induced adhesion. Although we could not detect erbB4 protein expression in our experiments with the MDA-MB-435 cells, it was possible that low, but undetectable levels of erbB4 might be mediating the HRG β effects that we observed. However, an antibody that blocks HRG β binding to erbB4 [14] did not inhibit HRG β -induced adhesion, even when combined with the erbB3 blocking antibody [1]. Although no effects of the erbB2 or erbB3 blocking antibodies were seen on EGF-mediated adhesion, only EGF-mediated adhesion was abrogated by the highly EGFR-specific inhibitor tyrphostin AG1478 [16] in a dose-dependent fashion (Figure 2). At a concentration of 50-100 nM inhibitor, the EGF-induced adhesion was reduced to approximately the levels of unstimulated cell adhesion with little effect observed on the ability of HRG β or TS2/16 to stimulate maximal adhesion.

Because of the strong stimulation of cell migration initiated by HRG β , we extended our antibody blocking studies to COLL and LAM migration assays to determine the receptor

subunits contributing to these signals. Similar to the adhesion assays, anti-erbB3 and anti-erbB2 antibodies blocked HRG β -stimulated MDA-MB-435 cell migration toward laminin or collagen [1]. In addition, incubation of cells with the tyrphostin AG1478 had negligible effects on either unstimulated or HRG β -stimulated cell migration (data not shown). In contrast, EGF-stimulated migration of MDA-MB-435 cells was not inhibited by the anti-erbB2 antibody [1]. To demonstrate that this anti-erbB2 antibody was in fact blocking EGF signaling, we explored the phosphorylation status of erbB2 in response to EGF or HRG β after exposure to the anti-erbB2 antibody. Both EGF and HRG β induced tyrosine phosphorylation of erbB2, and preincubation of cells with the inhibitory anti-erbB2 antibody dramatically reduced the activation of erbB2 tyrosine phosphorylation by both growth factors [1]. These results show that both EGF and HRG β result in increased phosphorylation of erbB2 in MDA-MB-435 cells. However, since the anti-erbB2 antibody blocks EGF-induced phosphorylation of erbB2 but not EGF-stimulated adhesion or migration, this suggests a differential role for erbB2 in EGF versus HRG β -mediated regulation of β 1 integrin function. An interesting picture of preferential partnering with erbB2 is suggested by these studies for EGFR- versus erbB3-mediated signaling of adhesion or migration. While erbB2 is a favored dimerization partner for the EGFR, even over EGFR-EGFR homodimers [17,18], we did not observe any significant inhibition of EGF-stimulated adhesion by the erbB2 antibody. This suggests that for cellular adhesion and migration, erbB2 may have more of a role as a conduit for erbB3-initiated signals, while the EGFR does not require erbB2 for the more moderate effects on adhesion in these cells.

Role of PI 3-K in EGF- and HRG β -induced upregulation of β 1 integrin function. As PI 3-K plays a role in signaling by the EGFR family members, we analyzed the contributions of this enzyme to the adhesion and migration events we had observed in the MDA-MB-435 cells. Stimulation of MDA-MB-435 cells with either EGF or HRG β results in rapid recruitment of the p85 subunit of PI 3-K to the phosphotyrosine-containing cellular fraction [1], consistent with previous reports of growth factor stimulated PI 3-K activation in other cell lines [19]. Analysis of PI 3-K activity from anti-phosphotyrosine immunoprecipitates further demonstrated the increased activity of PI 3-K in response to EGF or HRG β stimulation [1]. Consistent with the effects of EGF and HRG β on integrin-mediated adhesion and migration, HRG β induced more potent PI 3-K activity than EGF [1].

We tested the relevance of PI 3-K activation to adhesion with two pharmacologically distinct PI 3-K inhibitors, wortmannin and LY294002 [20-23]. When cellular adhesion assays were performed in the presence of either 100 nM wortmannin or 25 μ M LY294002, we observed a significant, although incomplete, decrease in both EGF- and HRG β -stimulated MDA-MB-435 cell adhesion to collagen [1]. In contrast, only small reductions in TS2/16-induced or unstimulated adhesion were observed. PI 3-K appears to contribute even more significantly to the process of migration in these cells as both inhibitors markedly reduced HRG β -mediated migration on laminin as well as collagen [1]. In these experiments, unstimulated migration was also reduced, but only at the highest doses of the inhibitors. While cells generally migrated in smaller numbers in experiments using EGF as a stimulus, motility induced by EGF on COLL and LAM was also strongly inhibited by wortmannin or LY294002 [1].

In order to further support our pharmacological inhibitor data, we employed a transient transfection assay that allowed us to assess the adhesion and migration of untransfected as well as transfected cells by flow cytometric analysis of adherent or migrated cell populations [24,25]. Control vector expressing green fluorescent protein (GFP) alone or constructs expressing GFP-tagged wild type p85 or a dominant negative p85 subunit (Δ p85) were transiently transfected into

MDA-MB-435 cells followed by analysis in a modified cell adhesion assay. Increasing levels of GFP alone (C2) had little effect on the adhesion of MDA-MB-435 cells under any stimulation condition, while expression of either wtp85 or $\Delta p85$ subunits of PI 3-K decreased EGF or HRG β -mediated adhesion by approximately 50% without affecting TS2/16 or unstimulated adhesion significantly [1].

The effects of molecularly inhibiting PI 3-K function on HRG β -stimulated cell migration of MDA-MB-435 cells was also investigated. Comparison of control transfected or $\Delta p85$ -transfected cell migration in standard Boyden chamber conditions revealed some inhibitory effect of $\Delta p85$ expression on HRG β -mediated cell migration, while no striking inhibition of unstimulated migration was apparent [1]. However, analysis of the specific GFP-positive, $\Delta p85$ -positive cells in comparison to GFP-negative cells showed a striking inhibition of both HRG β -stimulated and unstimulated cell migration with increasing expression of the $\Delta p85$ construct [1], in keeping with our results using wortmannin and LY294002. Thus, both EGF- and HRG β -stimulated pathways require functional PI 3-K for optimal $\beta 1$ integrin-mediated adhesion and migration of MDA-MB-435 cells.

Role of EGFR family members in regulating $\beta 1$ integrin function on other breast carcinoma cell lines. While the focus of the majority of our work was on MDA-MB-435 cells as a model system for growth factor regulation of integrin-mediated adhesion and migration, we made several compelling observations during the course of analyzing other cell lines in our studies. Our adhesion assays showed consistent EGF-induced increases in adhesion of the T47D cell line to collagen as well as FN (Figure 1E), both of which were completely blocked by inhibitory $\beta 1$ integrin-specific antibodies (Figure 3 A, B). However, two observations suggest that these cells are mechanistically distinct from the MDA-MB-435 cells. First, while adhesion to FN can be stimulated by EGF, betacellulin, or HRG β , only adhesion stimulated by the EGF family ligands (EGF and betacellulin) was sensitive to the PI 3-K inhibitors wortmannin and LY294002 (Figure 4). In contrast to our results with MDA-MB-435 cells [1], HRG β -induced adhesion of T47D cells was insensitive to both of these inhibitors (Figure 4). Second, while the EGFR family blocking antibodies were quite effective in elucidating the dimerization partners mediating erbB3 versus EGFR signaling in MDA-MB-435 cells, these same antibodies were completely ineffective at abrogating the EGF- or HRG β -stimulated adhesion of T47D cells to FN or collagen (Figure 3), even at 10X the typical dose of 1 μ g antibody/ 1×10^6 cells (Figure 3C and D). T47D cells express EGFR, erbB2, and erbB3 at levels similar to MDA-MB-435 cells, but with the additional expression of erbB4. However, erbB4-blocking antibodies were also not effective alone or in combination in reducing HRG β or EGF-stimulated adhesion of these cells (Figure 3A and B).

While both MDA-MB-435 and T47D cells express modest levels of the growth factor receptors we have examined, SKBR3 cells express relatively high levels of EGFR and erbB3. To our surprise, we observed no inhibition of HRG β or EGF-stimulated adhesion of SKBR3 cells to collagen or merosin by the anti-erbB3 or anti-erbB4 antibodies (Figure 5A, B). However, the anti-erbB2 blocking antibody appeared to effectively abrogate EGF-stimulated adhesion of SKBR3 cells to collagen or merosin without affecting HRG β -stimulated adhesion (Figure 5C and D). Although we often noted some increase of unstimulated adhesion in the presence of the erbB2 blocking antibody (compare Figure 3C and D and Figure 5C and D), EGF-stimulated adhesion was routinely reduced to the levels of unstimulated adhesion in the presence of the blocking antibody in at least three separate experiments. Thus, analyses of growth factor stimulated upregulation of $\beta 1$ integrin function across several cell lines has hinted at the

complexity in regulation both by signaling pathways utilizing PI 3-K, and in the receptor-receptor specificity of interactions that transmit signals from bound EGF or HRG β to the cellular network governing adhesion and migration.

Role of the Cbl adapter protein in regulation of breast cancer cell adhesion and migration. Recent work from our laboratory in the study of human lymphocytes has suggested a role for the 120 kDa Cbl adapter protein in β 1 integrin-mediated adhesion events [26]. In addition, Cbl has recently been implicated in the regulation of growth factor receptor signaling as demonstrated by its role in the ubiquitin-dependent downregulation of PDGF-receptors following growth factor stimulation [27,28]. These studies may be particularly relevant to our system given the observation that Cbl is primarily recruited to the EGFR and not to other members of the EGFR family [29] and that the EGFR is significantly downregulated in response to ligand binding while erbB2, erbB3, and erbB4 are not regulated in the same fashion [30,31]. Finally, studies in *C. elegans* [32] and *Drosophila* [33] suggest that Cbl is a negative regulator of the EGFR, and recent functional studies in mammalian cells have implicated the JAK/STAT pathway rather than the raf pathway in mediating Cbl's regulatory effect on the EGFR [34]. This is consistent with our preliminary data showing little effect of a MEK inhibitor on EGF or HRG β -mediated adhesion or migration (data not shown).

In EGFR signaling pathways, Cbl becomes highly phosphorylated in response to EGF, can be shown to coprecipitate with the EGFR, and is believed to participate in the downregulation of this receptor following ligand binding. In contrast, Cbl is apparently not a participant in erbB3 mediated signaling as assessed by its lack of phosphorylation and absence in erbB3 receptor immunoprecipitates following HRG β stimulation. However, Cbl is known to interact with PI 3-K, a molecule important for both EGF and HRG β -mediated adhesion and migration in MDA-MB-435 cells. In addition, the role of Cbl in the cross-talk between the EGFR or erbB3 and β 1 integrins remains unexplored. In order to ascertain the possibility that Cbl might play a differential role in EGF and HRG mediated adhesion and migration in MDA-MB-435 cells, we first biochemically analyzed Cbl in response to growth factor stimulation when cells were additionally stimulated through binding to COLL-coated plates (Figure 6). Tyrosine phosphorylation of a 120 kDa protein (open arrow) from Cbl immunoprecipitates was rapidly induced by exposure of cells to EGF, with a maximal signal at 1 minute of stimulation and a reduced but sustained tyrosine phosphorylation to 60 minutes of stimulation at 37°C. An apparent 170 kDa tyrosine phosphorylated protein was visible in Cbl immunoprecipitates whose presence followed the same general time course as that of Cbl tyrosine phosphorylation. (Figure 6, solid arrow). In contrast, cells plated onto COLL and subsequently exposed to HRG β for various periods of time also revealed increased tyrosine phosphorylation of a 120 kDa protein. However, the time course of stimulation was unique from that of EGF, with maximal signal intensity occurring at 15 minutes and a more diminished signal thereafter. In addition, a 180 kDa tyrosine phosphorylated protein was observed coprecipitating with Cbl between 5 and 60 minutes of stimulation, with maximal signal at 15 minutes of HRG β stimulation (Figure 6, flanged arrow). Subsequent immunoblotting with anti-Cbl demonstrated the 120 kDa protein to be Cbl and reflected similar levels of Cbl immunoprecipitated in each lane (data not shown). The molecular weights of the 170 and 180 kDa proteins suggest their possible identities to be the EGFR and erbB3, respectively.

In order to examine the significance of adhesion to the tyrosine phosphorylation of Cbl in response to EGF and HRG β , we compared Cbl immunoprecipitates from MDA-MB-435 cells that were stimulated in suspension (Figure 7, upper panel) or on COLL-coated plates (Figure 7,

lower panel) over time. Tyrosine phosphorylation of Cbl was rapidly induced by exposure to either EGF or HRG β in suspended cells, with the most intense signal between 1 and 5 minutes of stimulation. For each growth factor there was a lower, but sustained phosphorylation of Cbl for the remaining time points, but the intensity of Cbl phosphorylation in response to HRG β was comparatively smaller than that seen by EGF stimulation. In addition, striking coprecipitation of the 170 kDa protein was present in EGF stimulated lanes, but no apparent 180 kDa band was observed in HRG β stimulated lanes. Adhesion to COLL followed by growth factor exposure showed an enhanced and sustained tyrosine phosphorylation of Cbl and coprecipitation of the 170 kDa protein in response to EGF (Figure 7, lower panel). As seen in Figure 6, the signal intensity of both Cbl and the 170 kDa protein bands were more sustained when cells were stimulated both through β 1 integrins binding to COLL and exposure to EGF. While the EGF response followed a similar time course in each case, the addition of HRG β to cells plated onto COLL was markedly different than the time course seen in suspension. Specifically, low levels of Cbl phosphorylation were seen at early time points, but maximal Cbl phosphorylation was evident at late, rather than early time points, namely at 30 and 60 minutes of stimulation. As seen in Figure 6, the coprecipitation of a 180 kDa protein was observed in Cbl immunoprecipitates reflecting the highest tyrosine phosphorylation in response to HRG β .

Collectively, Figures 6 and 7 suggest that not only EGF, but also HRG β can stimulate Cbl tyrosine phosphorylation in MDA-MB-435 cells and that the Cbl phosphorylation in response to either growth factor is distinct in suspended versus cells plated onto COLL, the latter receiving both β 1 integrin and growth factor signals. In addition, our preliminary data indicate the possibility of coprecipitation of Cbl not only with the EGFR, as has been previously reported, but also possibly with erbB3 in these cells, in an adhesion-specific fashion.

To assess the functional consequence of differential signaling from the EGFR or erbB3 to influence the tyrosine phosphorylation of Cbl, we have transiently expressed a GFP-tagged wild type Cbl (GFP-Cbl) in the MDA-MB-435 cells followed by adhesion (Figure 8) and migration (Figure 9) assays. Analysis of GFP- or GFP+ cells that were stimulated to adhere to COLL by EGF suggested that increasing expression of Cbl reduced the EGF-stimulated adhesion (panel 8A) while having little effect on HRG β -mediated adhesion (Panel 8B). Similarly, when GFP- and GFP+ cells were analyzed by flow cytometry following migration on LAM, the overexpression of Cbl was seen to inhibit EGF-mediated migration (Figure 8A), but little influence was seen on HRG β -mediated migration (Figure 8B). However, it is important to emphasize that these results are very preliminary and will require additional experiments in the future. We have developed numerous Cbl mutants that could be easily tested in this system for their potential to modulate EGF- or HRG β -induced adhesion and migration.

Role of R-ras in regulation of breast cancer cell adhesion and migration. Additional preliminary data from our laboratory suggest a regulatory role for the R-ras molecule on the adhesion of lymphocyte cell lines (unpublished observations). R-ras has transforming properties in certain cell types [35,36] and couples to some of the same signaling pathways as H-ras. However, it has also shown unique regulation in, for example, its inability to couple to the Sos regulatory molecule or to activate Raf [37-40]. While little is yet known about the contributions of R-ras to cellular adhesion and migration pathways, at least one report has suggested a regulatory role for R-ras on integrins [41]. A recent report has also implicated R-ras as an important participant in cellular motility, demonstrating increased cell scattering of epithelial cells in the presence of constitutively active R-ras [42]. Importantly, PI 3-K mediates the primary pathway involved in this response, utilizing downstream effectors distinct from rac and

Akt. This is also consistent with studies demonstrating that R-ras can activate PI 3-K but not MAP kinase [37]. We have wild-type and mutant forms of R-ras available as GFP fusion proteins that we will test to see if they alter growth factor-mediated regulation of $\beta 1$ integrin function.

In preliminary studies, we examined the effect of overexpressing R-ras on MDA-MB-435 cell adhesion and migration. Because very high transient expression was achieved with DNA constructs expressing wt R-ras (wt), a constitutively active mutant (V38), or a dominant negative mutant (N43), bulk transfectants were used in standard adhesion (Figure 10A) and migration (Figure 10B) assays. Stimulation of cells with the activating TS2/16 monoclonal antibody induced strong adhesion in untransfected (null) as well as all transfected cell populations (Figure 10A). EGF and HRG β stimulation also caused increased adhesion, as we have previously demonstrated. However, expression of wt R-ras or its mutants, even at high levels of expression, resulted in minimal effects on the stimulated adhesion we observed. Migration of null transfected and all transfected cells was induced by both EGF and HRG β stimulation. Although EGF-induced migration was unaffected by overexpression of R-ras, there was a slight enhancement of HRG β -induced migration in the presence of wild-type or mutant R-ras. Similar data were obtained by performing transient-transfection assays followed by flow cytometric analysis of GFP- and GFP+ adherent or migrated cell populations (data not shown).

6.C. RECOMMENDATIONS RELATED TO ORIGINAL PROPOSAL

In the first aim of this IDEA Award, we proposed analyzing several parameters related to breast cancer cell adhesion, motility, and invasion. We have accomplished the objectives outlined in the original proposal and generated a body of compelling data that has helped to redefine key points remaining to be addressed within the context of this proposal. First, we proposed screening a variety of cell lines for integrin and growth factor receptor expression, to assess the activation of PI 3-K in these cell lines, and to examine the nature of their adhesion to various ECM ligands. Using FACS and blotting analyses, we characterized the integrin and EGFR family profiles of several breast cancer cell lines. Preliminary adhesion data revealed a subset of breast cancer cell lines that were informative from the standpoint of upregulated adhesion in response to growth factor stimulation. We further characterized those lines, including MDA-MB-435, T47D and SKBR3 cells. We observed activation of PI 3-K upon EGF or HRG β stimulation of MDA-MB-435 cells, and specifically observed more potent activation of PI 3-K by HRG β compared to EGF [1]. We have not been able to examine each cell line for PI 3-K activation in response to EGF or HRG β due to the desire to focus our efforts on a more narrowly-defined system. However, these studies are clearly important to pursue in the future since there are notable differences in sensitivity of adhesion and migration responses to wortmannin and LY294002 in some of these cell lines.

We also examined adhesion events to several ECM ligands, including FN, type IV collagen, merosin, and laminin. While we could demonstrate growth factor-induced adhesion and migration on all of these ECM components to differing degrees, we focused our efforts on $\beta 1$ integrin-dependent adhesion of MDA-MB-435 cells to type IV collagen, in the hopes of more clearly understanding the repertoire of molecules interacting in the context of a single cell type. In addition, we have demonstrated the dramatic upregulation of breast cancer cell adhesion and migration in response to treatment with the erbB3 and erbB4 ligand, HRG β , and have described the preferential recruitment of erbB2 to erbB3 heterodimers over the EGFR when signals are initiated from HRG β or EGF, respectively. This was a new finding that was not expected in the

original aim. In fact, our studies suggest a particularly critical role for HRG β in regulating breast cancer cell adhesion and migration. This is one of the most significant outcomes of our research.

The second aim of this proposal was to examine the role of PI 3-K in EGF-regulation of β 1 integrin function. Using the two approaches that were originally proposed, we have demonstrated a clear role for PI 3-K in not only the EGF upregulation of integrin-mediated adhesion and migration but also those stimulated by HRG β . Instead of an inducible expression system as originally suggested for expression of dominant negative forms of PI 3-K, we employed transient expression of PI 3-K mutants combined with modified adhesion [24,25] and migration assays and analysis of specific cell populations by flow cytometry as a means of supporting the pharmacological data we had generated. We have demonstrated inhibition of EGF- and HRG β -stimulated MDA-MB-435 cell adhesion and migration by dominant-negative p85 using this method.

While evidence exists that PI 3-K is involved in unstimulated invasion by MDA-MB-435 cells [43], our work has now demonstrated that EGFR family members also play an important role in regulating adhesion and migration mediated by β 1 integrins. Our studies are consistent with other recent studies demonstrating a role for PI 3-K in EGF-induced migration of MDA-MB-231 breast cancer cells [44]. However, our results are in contrast to another study that analyzed breast epithelial cell migration in response to EGF [45]. In this report, transfectants overexpressing the EGFR showed a dramatic increase in EGF-stimulated cell migration in comparison to parental ZR75-1 cells. Incubation with wortmannin and LY294002 enhanced EGF-mediated migration of EGFR-overexpressing cells, while the MEK1 inhibitor PD98059 decreased EGF-stimulated adhesion. However, no effect of either inhibitor was observed on the parental cells, reported to express approximately 20,000 EGFR on the cell surface as compared to 1,200,000 EGFR on the transfected cells. Our results suggest that PI 3-K plays a positive role in both stimulated and unstimulated adhesion and migration of MDA-MB-435 cells, and we have noted little effect of inhibitors affecting the MAPK pathway (data not shown), as reported by others [43]. Altogether, these observations underscore the need to further clarify the role of the PI 3-K pathway and to understand its contributions to the signals generated by a given repertoire of EGFR family receptors expressed on a given cell type.

In addition, we have made preliminary observations in a second cell line, T47D, of differential regulation of EGF and HRG β -driven pathways, exemplified by differing sensitivities to the PI 3-K inhibitors wortmannin and LY294002. While T47D cells express similar levels of EGFR, erbB2, and erbB3 to those found in MDA-MB-435 cells, they express the additional HRG β receptor, erbB4, which is not expressed on MDA-MB-435 cells. Recent reports have described unique proteolytic processing of erbB4 [46-48] as well as at least one alternate transcript, found in both normal breast tissue and in several breast tumors, that lacks a putative p85 binding site [49]. The relevance of this observation with regard to our studies is currently unclear but warrants further investigation. Divergent signals generated from ligand binding to erbB3 compared to the EGFR appears likely, given the unique nature of the erbB3 cytoplasmic tail, which recruits a unique panel of phosphoproteins compared to that of the EGFR. For example, erbB3 does not bind to PLC γ or GAP [50], and preferentially interacts with Grb7 rather than Grb2 [51]. ErbB3 also contains at least 6 sites in its cytoplasmic tail that are considered to be optimal for binding the SH2 domain of p85. In addition, varying reports have described mitogenic, growth-inhibitory, or differentiative effects of HRG stimulation depending on the growth factor isotype, the concentration used and the cell line studied [17,52-54]. Thus, these studies on erbB3 and erbB4 signaling upon HRG binding reveal the need for a better

understanding of their contributions to a variety of cellular processes, including cell adhesion and migration.

Our initial proposal outlined additional experimental avenues that remain for future endeavors. These include the examination of growth factor stimulation on affinity and avidity changes of $\beta 1$ integrins as well as invasion studies to further our observations of cell migration. In addition, we believe that further study is warranted to pursue the observation we have made regarding differential sensitivity of EGF or HRG β -mediated pathways in at least one cell line to PI 3-K inhibition. Given the current complexity of the EGFR family of receptors and their roles in the various cellular processes contributing to tumor cell formation and metastatic growth, it will be important to more clearly delineate the dimerization events undertaken by members of the EGFR family following the binding of specific ligands in a way that is informative for the signals generated by those ligands to upregulate $\beta 1$ integrins. Finally, several additional molecules of interest could be investigated for their contribution to EGF or HRG β -mediated pathways. As described above, we have initiated several new studies to explore the role of the Cbl adapter protein and the R-Ras protein in EGF- and HRG β -induced regulation of breast cancer cell adhesion and migration.

Finally, the contributions of Rho-family proteins such as rac1 and cdc42 to cellular cytoskeletal changes such as membrane ruffling and lamellipodia formation have been appreciated for some time [55], but their placement in signaling pathways between growth factor receptors and integrin activation is relatively unexplored. These signaling molecules, and various dominant negative or constitutively active forms, are currently available for our use, most being based in a vector to produce GFP-fusion proteins, lending themselves well to our transient expression-adhesion and migration assays. Thus, we will begin exploring the potential role these molecules may have in EGF or HRG β signaled events to $\beta 1$ integrins in breast carcinoma cells and to determine their contribution to the PI 3-K pathways initiated by growth factor binding to the EGFR family of receptor tyrosine kinases.

7. KEY RESEARCH ACCOMPLISHMENTS

- Growth factors can bind and activate epidermal growth factor receptors (EGFRs) to upregulate cellular adhesion and migration via the activation of $\beta 1$ integrins
- Growth factor upregulation of $\beta 1$ integrins is mediated through the activation of phosphoinositide 3-kinase.
- HRG β is a more potent activator of $\beta 1$ integrin-mediated adhesion and migration and PI 3-K activity than EGF
- The erbB2 receptor preferentially participates in HRG β -, but not EGF-, stimulated adhesion and migration of breast carcinoma cells

8. KEY REPORTABLE OUTCOMES

MANUSCRIPTS AND ABSTRACTS

1. Adelsman, M.A., McCarthy, J.B. and Shimizu, Y.: Stimulation of $\beta 1$ integrin function by epidermal growth factor and heregulin β has distinct requirements for erbB2 but a

similar dependence on phosphoinositide 3-OH kinase. *Mol. Biol. Cell* 1999; 10:2861-2878.

2. Adelsman, M.A., Vachhani, B.V., McCarthy, J.B., and Shimizu, Y.: Heregulin stimulation of breast carcinoma cell adhesion and migration is mediated by $\beta 1$ integrin and requires PI 3-kinase. *Mol. Biol. Cell* 9:115a. American Society for Cell Biology Annual Meeting, December 12-16, 1998.

FUNDING APPLIED FOR BASED ON WORK SUPPORTED BY THIS AWARD

U.S. Army Medical Research (BC991135). IDEA Award proposal entitled "Function of Etk in growth factor receptor signaling to integrin in breast cancer cells. Review is pending.

PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

Yoji Shimizu, Ph.D.: Principal Investigator
James B. McCarthy, Ph.D.: Co-investigator
Margaret Adelsman, Ph.D.: Postdoctoral Associate
George Ballato: Scientist

9. CONCLUSIONS

Our data have clearly shown the ability of growth factors that bind to and activate the EGFR family of receptor tyrosine kinases to upregulate cellular adhesion and migration processes via activation of $\beta 1$ integrins. We have shown that that EGF and betacellulin, ligands that activate the EGFR, and HRG β , a member of the neuregulin family of ligands that bind to and activate erbB3 and erbB4, induce dose- and time-dependent adhesion of MDA-MB-435 cells to type IV collagen. This adhesion is mediated by $\beta 1$ integrins, and functional PI 3-K is required. These same growth factors also upregulate $\beta 1$ integrin-mediated migration on collagen and laminin, again utilizing PI 3-K-dependent pathways. Studies with blocking antibodies have further suggested a differential recruitment of erbB2 to the erbB3 receptor rather than to the EGFR upon growth factor binding. In other breast carcinoma cell lines, there are differences in the requirement for PI 3-K in EGF versus HRG β -mediated adhesion pathways. Further, the contributions by various EGFR family receptor subunits to EGF or HRG β signaling across different cell lines may be unique. The molecular basis for these differences is under investigation. Thus, our work over the past year has confirmed the validity of our initial hypothesis regarding the regulation of $\beta 1$ integrins by EGFR family members in breast cancer, and the role of PI 3-K in this signaling cascade. Our work has provided evidence for a previously unappreciated role for growth factor receptors in breast cancer cell motility and invasiveness. Furthermore, it has highlighted the functional relevance of activation-dependent regulation of integrin receptors on breast cancer cells, a relatively unexplored area in the breast cancer field. Finally, this proposal has provided new insights into the intracellular signaling pathways by which growth factors and integrin receptors interact to regulate the motile and invasive behavior of breast cancer cells. Elucidation of these signaling pathways will ultimately result in the identification of intracellular targets that could be used to regulate the motile and invasive behavior of breast cancer cells independent of the type of integrin receptor expressed on the cell surface. The challenge in the future will be to determine the signaling pathways downstream of PI 3-K that regulate $\beta 1$ integrin-mediated adhesion and migration of breast cancer cells.

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11. APPENDICES

11.A. FIGURE LEGENDS

Figure 1. EGF modulates breast carcinoma cell adhesion to ECM ligands. MDA-MB-435 (A), A431 (B and C), SKBR3 (D), MDA-MB-231 (E), or T47D cells (F) were analyzed for their ability to respond to EGF treatment in cell adhesion assays performed on fibronectin (FN), type IV collagen (COLL), merosin (MER), or mouse EHS-laminin (EHS-LAM). Cells that had been serum starved for 12-24 hours were harvested by release from tissue culture flasks in 1 mM EDTA. Suspended cells were then washed in serum-free media to remove excess EDTA prior to quantitation and labeling with Calcein AM as previously described [56]. Adhesion assays were performed in 96-well plates that were precoated overnight at 4°C with extracellular matrix (ECM) ligands as indicated. ECM proteins were coated at 1 µg/well unless otherwise noted. Cells were then added to wells containing assay media alone or stimulators: the activating β 1 integrin-specific monoclonal antibody, TS2/16, or EGF at 100 ng/ml final concentration or at increasing amounts as indicated (B and C). Cells were allowed to settle briefly in wells before analyzing preadherent fluorescence on a fluorescence plate reader. Cells were then stimulated at 37°C for 10 minutes before removing nonadherent cells by hand washing with a syringe/manifold system. Cells plated on BSA alone as a control for nonspecific adhesion generally showed less than 10% adhesion (data not shown). Comparison of adhesion to human type IV collagen or mouse EHS-collagen, or human laminin and mouse EHS-laminin did not show significant differences (data not shown).

Figure 2. Effects of the EGFR-specific tyrphostin AG4178 on EGF and heregulin- β mediated adhesion of MDA-MB-435 cells to collagen. Adhesion assays were performed as previously described [1]. EGF signaling was examined by stimulating cells with PBS, TS2/16, EGF, or HRG β in the presence of increasing amounts of the EGFR-specific tyrphostin AG1478 or DMSO control.

Figure 3. T47D cell adhesion to FN or COLL is increased by stimulation with EGF or HRG β and is β 1 integrin-dependent, but cannot be abrogated by anti-erbB2, anti-erbB3, or anti-erbB4 blocking antibodies. Subconfluent cultures of T47D cells were serum-starved for 24 hours and labeled with Calcein AM following harvesting with 1 mM EDTA as described previously. Adhesion assays were carried out in 96-well plates that had been precoated overnight at 4°C with 1 µg/well FN (A and C) or 1 µg/well COLL (B and D). Blocking antibodies recognizing β 1 integrins (P5D2), erbB2, erbB3, or erbB4 were incubated with cells at 1 µg/1 X 10⁶ cells or at increasing amounts as indicated. Cells were then stimulated to adhere in the presence or absence of 1 µg/well TS2/16, 100 ng/ml EGF, or 100 ng/ml HRG β . Adherent cells were quantitated by fluorescence following plate washing as previously described.

Figure 4. Stimulated adhesion of T47D cells to FN mediated by EGF-family ligands or HRG β displays differential sensitivity to PI 3-K inhibitors. T47D cells were grown to subconfluency and serum-starved for 12 hours in RPMI 1640. Adhesion assays using Calcein AM labeled cells were carried out in the presence or absence of 10 ng/ml betacellulin (BCELL), 100 ng/ml EGF, or 100 ng/ml HRG β . Additionally, cells were exposed to either control DMSO, wortmannin (100 nM), or LY294002 (25 µM) for 10 minutes at 37°C prior to removal of nonadherent cells and quantitation of fluorescence in the adherent cell populations.

Figure 5. SKBR3 breast carcinoma cell adhesion to COLL or MER is increased by EGF or HRG β stimulation and is β 1 integrin-dependent, but only EGFR-activated signals require heterodimerization with erbB2. Subconfluent cultures of SKBR3 cells were serum-starved overnight and harvested in 1 mM EDTA. Cells were quantitated, labeled with calcein AM, and incubated on ice with blocking antibodies recognizing β 1 integrins (P5D2), erbB2, erbB3, or erbB4. Antibody-coated cells were then added to 96-well plates coated with 0.3 μ g/well collagen (A and C) or 0.3 μ g/well merosin (B and D) in the presence or absence of TS2/16, EGF, or HRG β .

Figure 6. EGF and HRG β stimulate tyrosine phosphorylation of Cbl in MDA-MB-435 cells plated onto COLL. Serum-starved MDA-MB-435 cells were harvested at subconfluence, and equal numbers of cells were allowed to settle for 15 minutes onto plates that had been coated overnight at 4°C with EHS-COLL. The indicated growth factors (100ng/ml) were then added to the cells on the COLL-coated plates and stimulated at 37°C for the indicated periods of time. Control cells were held at 37°C for the maximum stimulation time in the absence of growth factors (- lanes). All cells were lysed at the end of their incubation times, and clarified supernatants were immunoprecipitated with polyclonal anti-Cbl and Protein A beads. Washed immunoprecipitates were separated by SDS-PAGE and immunoblotted with 4G10 to detect phosphotyrosine. A time point of zero reflects the addition of growth factor on ice followed by immediate lysis. The presence of Cbl is indicated with the open arrow, and the presence of 170 kDa and 180 kDa coprecipitated proteins is reflected by the solid or flanged arrows, respectively.

Figure 7. EGF and HRG β stimulation of Cbl tyrosine phosphorylation is enhanced by the dual presence of growth factors and β 1 integrin stimulation. MDA-MB-435 cells were prepared as for Figure 6, except that parallel aliquots were prepared for stimulation at 37°C either while in suspension (upper panel) or after settling onto COLL-coated plates (lower panel). Stimulation was carried out for the times indicated, and cells were lysed and immunoprecipitated with anti-Cbl antibodies as before. SDS-PAGE gels of immunoprecipitates were transferred to PVDF membranes and immunoblotted for anti-phosphotyrosine with monoclonal antibody 4G10. The presence of Cbl (open arrow) and of 170 kDa and 180 kDa coprecipitated proteins (solid or flanged arrows, respectively) is indicated.

Figure 8. Effects of Cbl over-expression on EGF- and HRG β -stimulated adhesion of MDA-MB-435 cells to COLL. MDA-MB-435 cells were transiently transfected with vectors expressing GFP or GFP-Cbl, as previously described. Adhesion assays of transfectants in the presence or absence of EGF (Panel A) or HRG β (Panel B) were carried out, and adherent cells were specifically analyzed and quantitated by flow cytometry as described in Materials and Methods (unstimulated, open bars; TS2/16, grey bars; EGF (Panel A) or HRG β (Panel B), black bars).

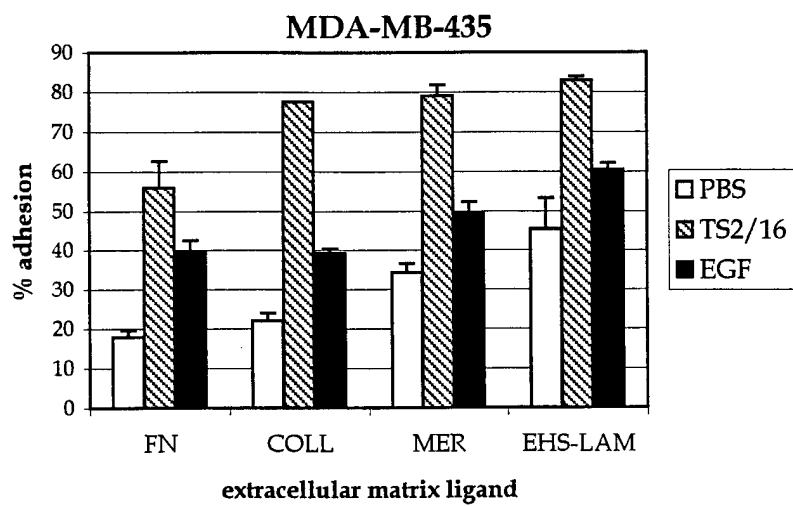
Figure 9. Effects of Cbl over-expression on EGF- and HRG β -stimulated migration of MDA-MB-435 cells on LAM. MDA-MB-435 cells were transiently transfected with vectors expressing GFP or GFP-Cbl, and the migration of bulk transfectants through LAM-coated transwells was assessed in the presence or absence of EGF (Panel A) or HRG β (Panel B). Migrated cells were trypsinized from the bottom of transwell filters and were specifically analyzed and quantified by flow cytometry as described in Materials and Methods (unstimulated, open bars; EGF (Panel A) or HRG β (Panel B), black bars).

Figure 10. Effects of wild-type and mutant R-ras on MDA-MB-435 adhesion and migration. MDA-MB-435 cells were transiently transfected with vectors expressing GFP, GFP-wt R-ras, GFP-N43 R-ras, or GFP-V38 R-ras, as previously described. GFP expression was assessed in bulk transfectants by flow cytometry and was approximately 60% for GFP alone and 85% for all R-ras-expressing constructs (not shown). Standard adhesion to COLL (Panel A) and migration on LAM (Panel B) were performed with bulk transfected cells in the presence or absence of various stimuli (unstimulated, open bars; TS2/16, grey bars; EGF, hatched bars, and HRG β , black bars). Results are shown in comparison to untransfected cell populations (null).

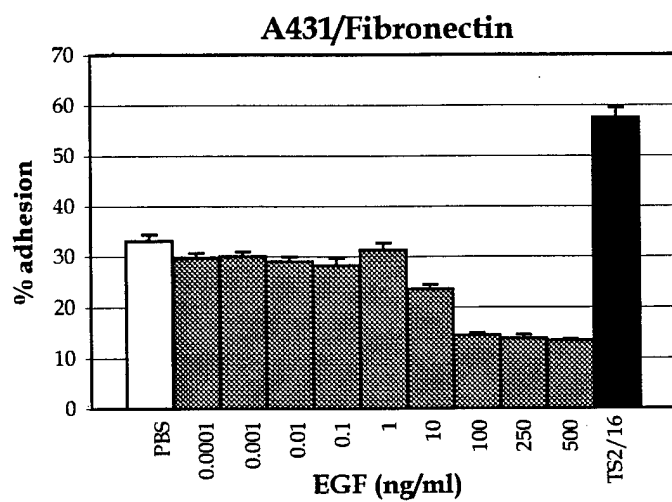
11.B. PUBLICATION

Adelsman, M.A., McCarthy, J.B. and Shimizu, Y.: Stimulation of β 1 integrin function by epidermal growth factor and heregulin β has distinct requirements for erbB2 but a similar dependence on phosphoinositide 3-OH kinase. *Mol. Biol. Cell* 1999; 10:2861-2878.

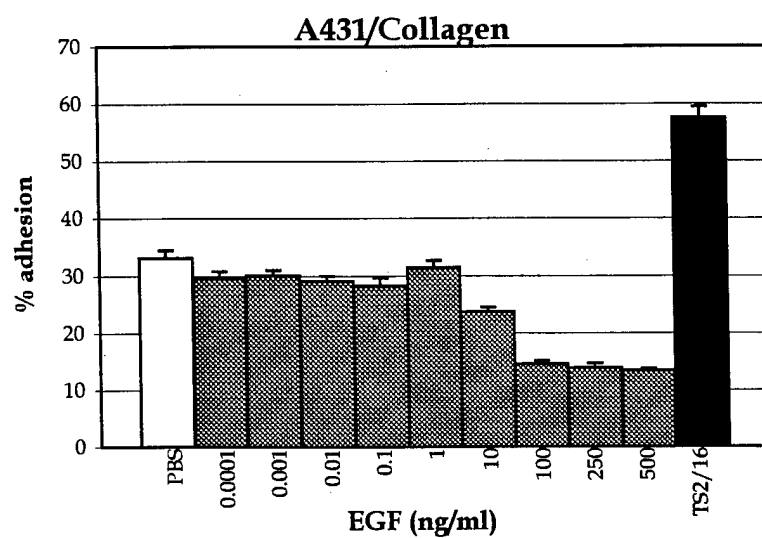
FIGURE 1



A

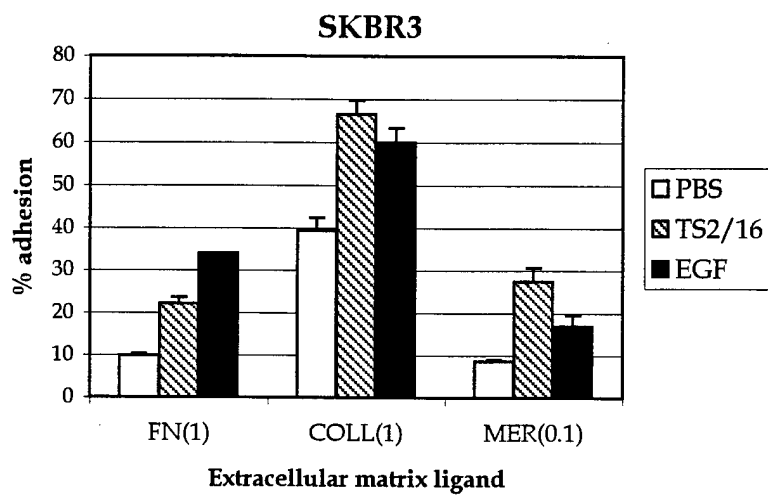


B

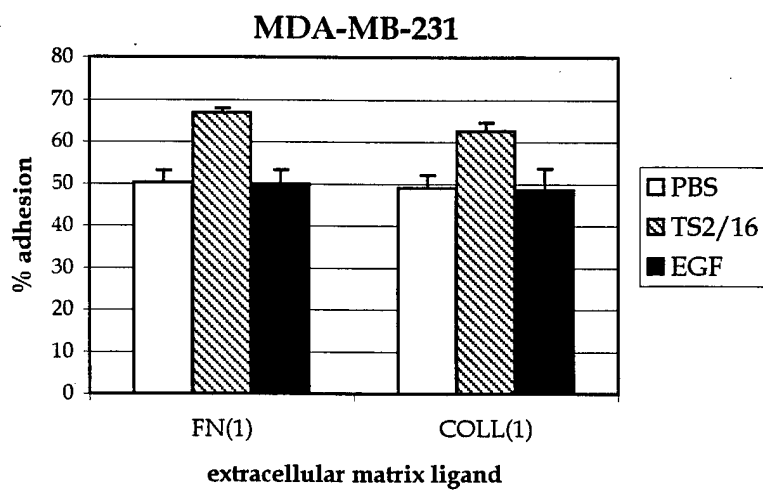


C

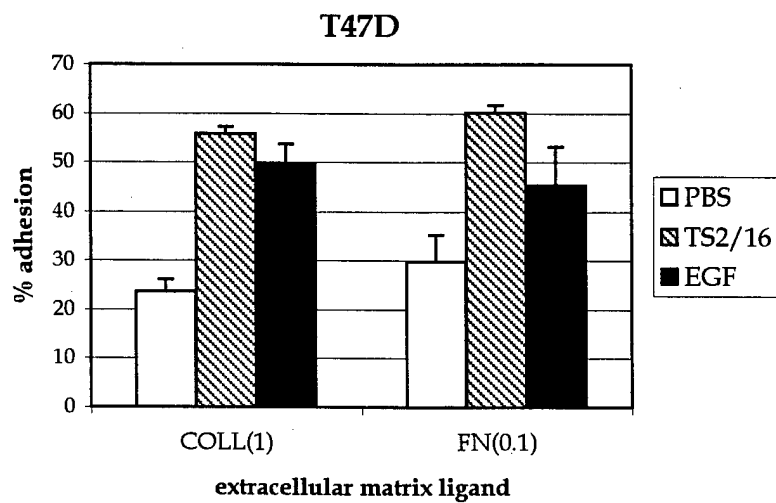
FIGURE 1



D

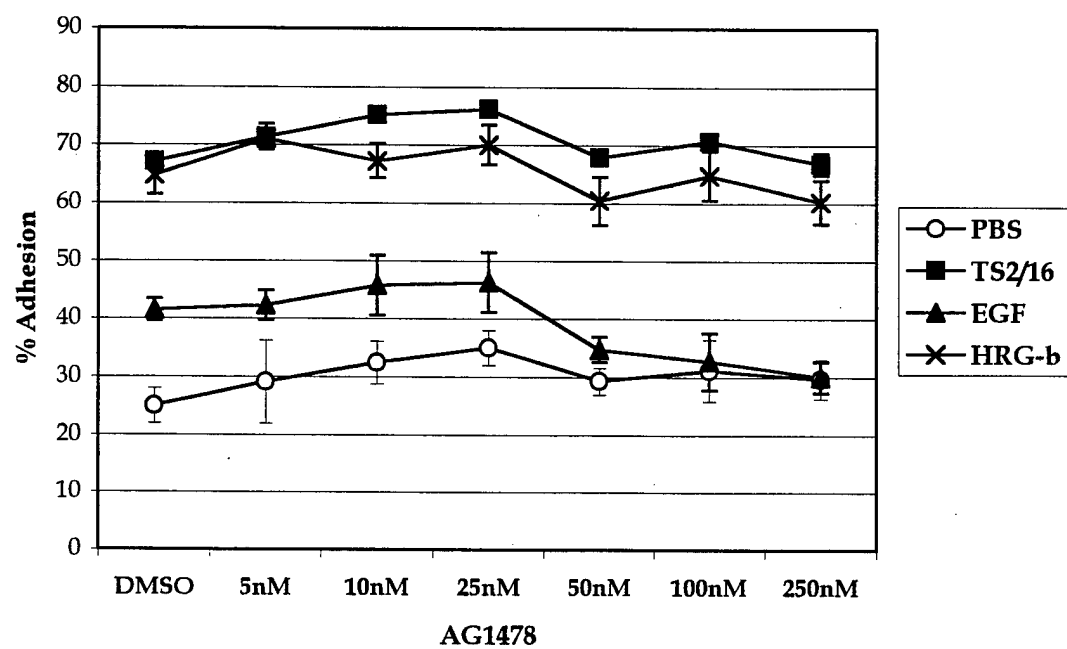


E



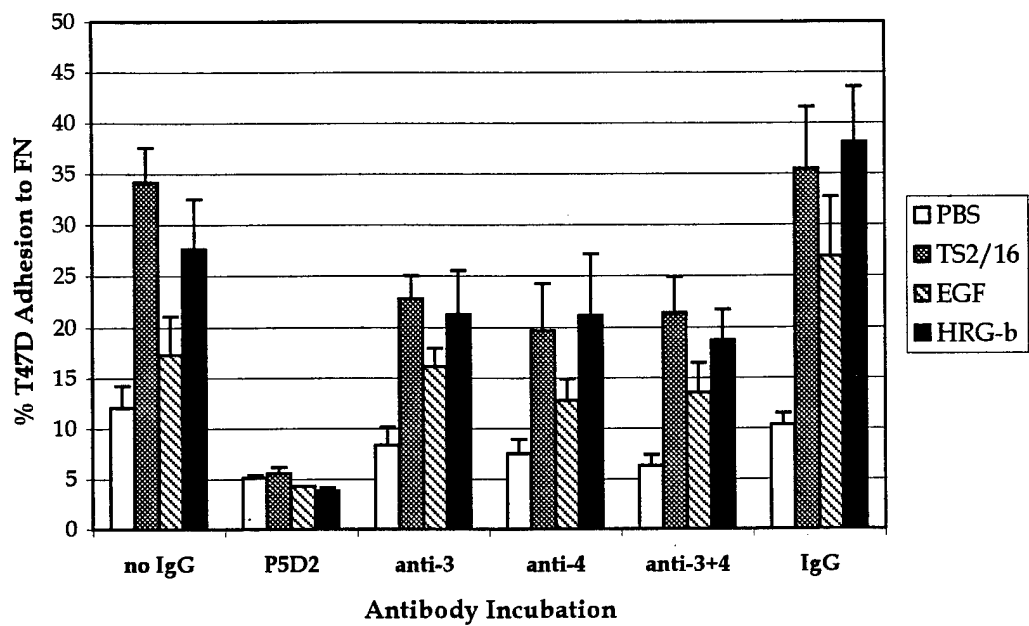
F

FIGURE 2

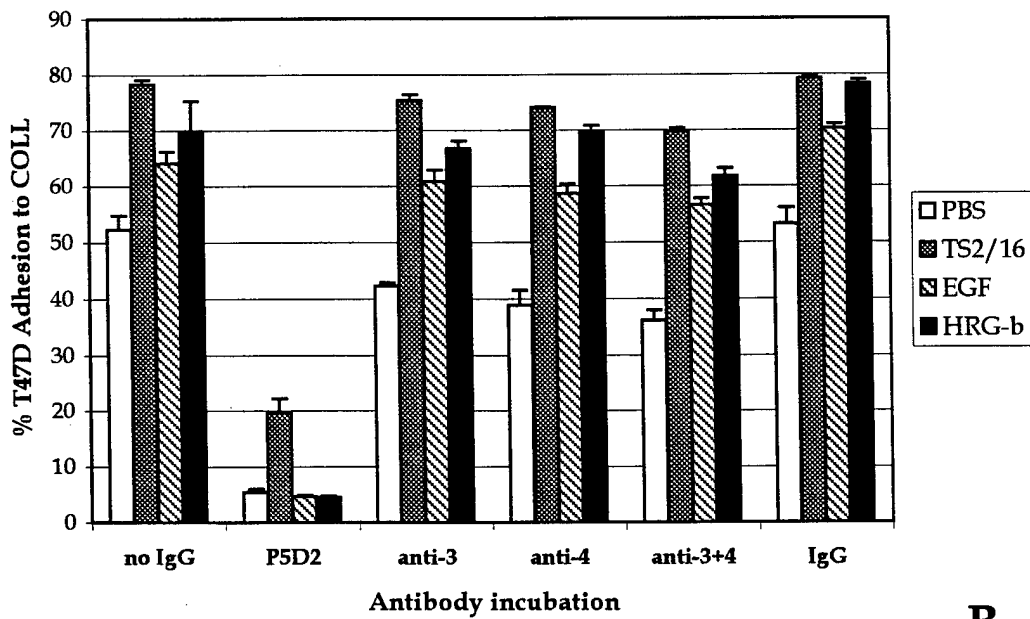


C

FIGURE 3

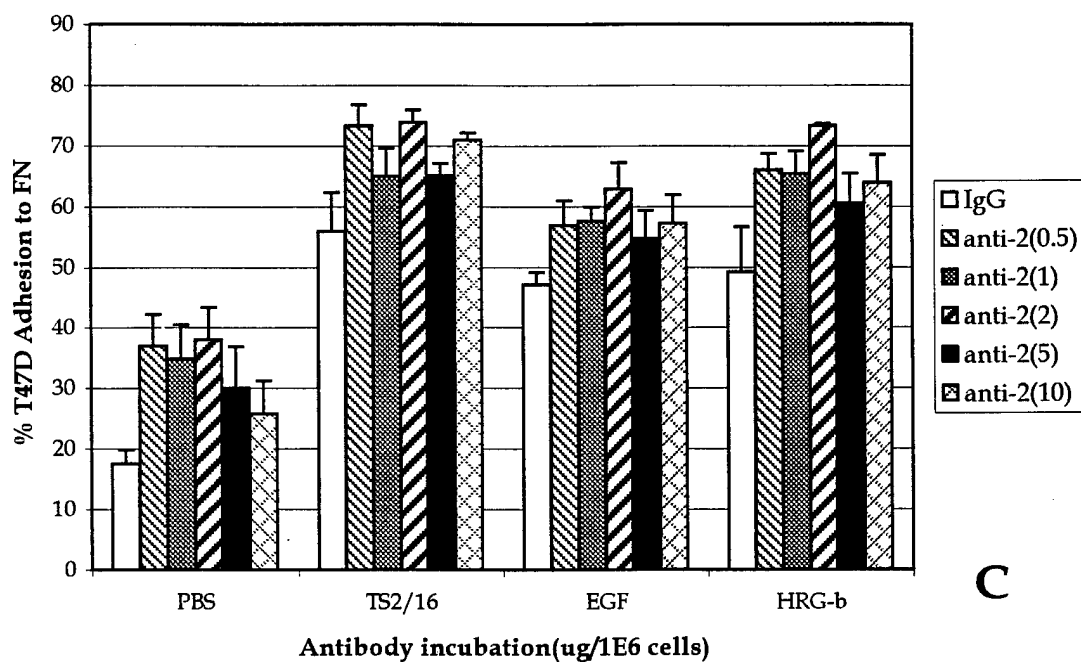


A

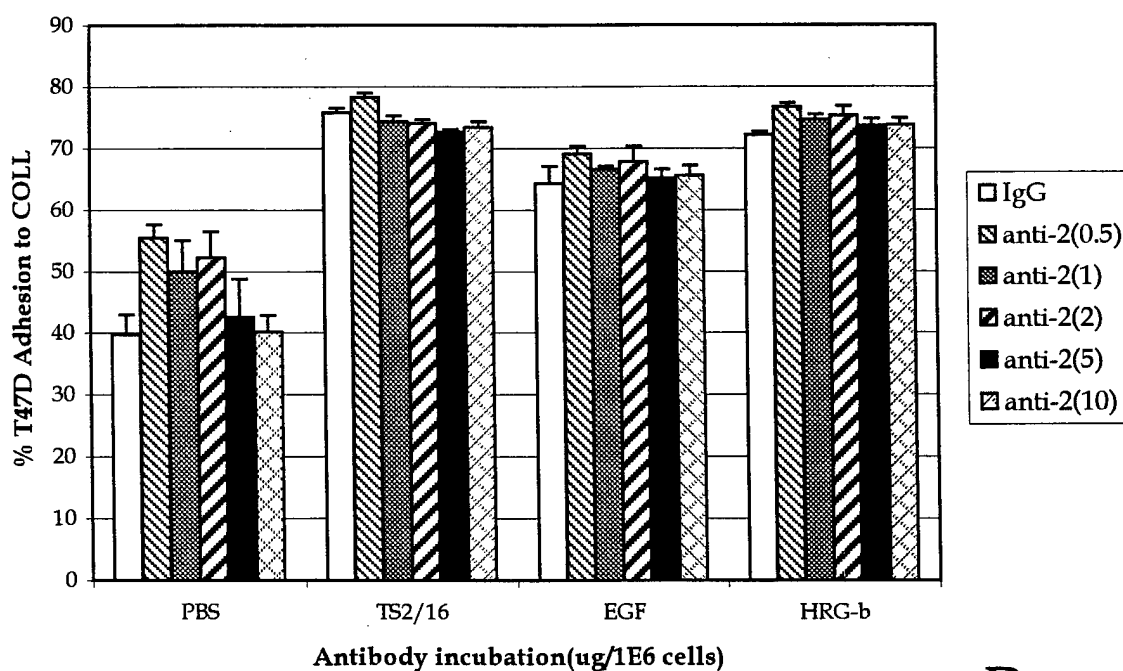


B

FIGURE 3



C



D

FIGURE 4

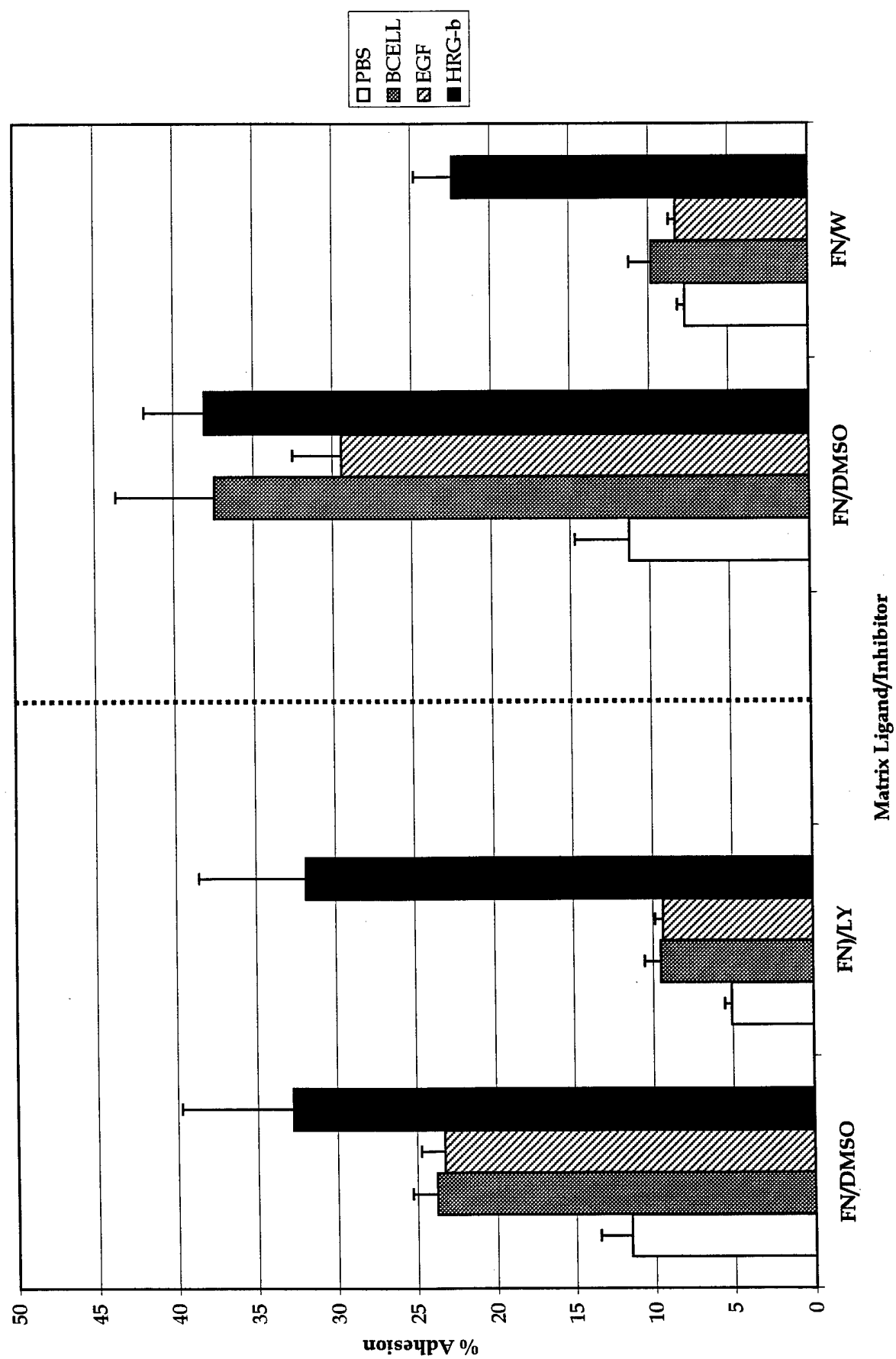
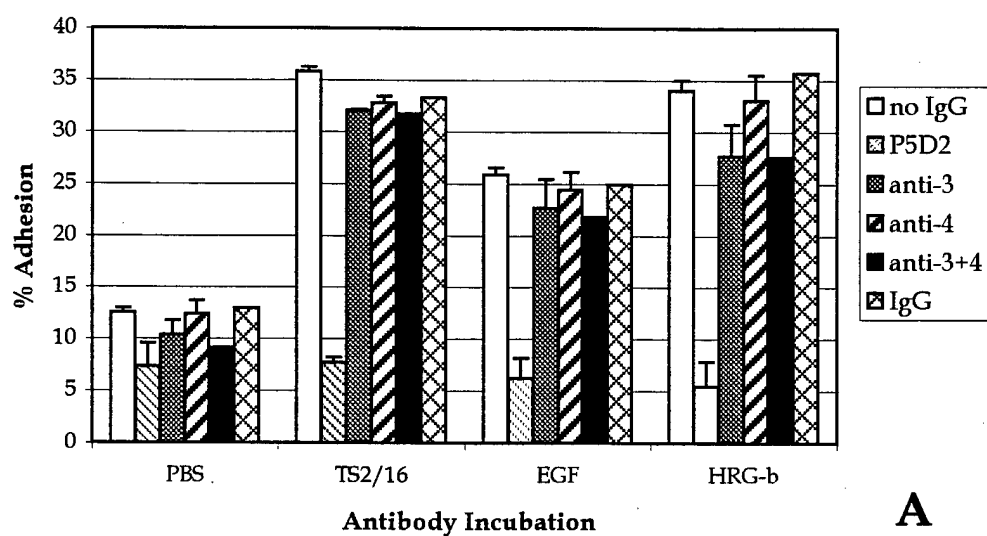
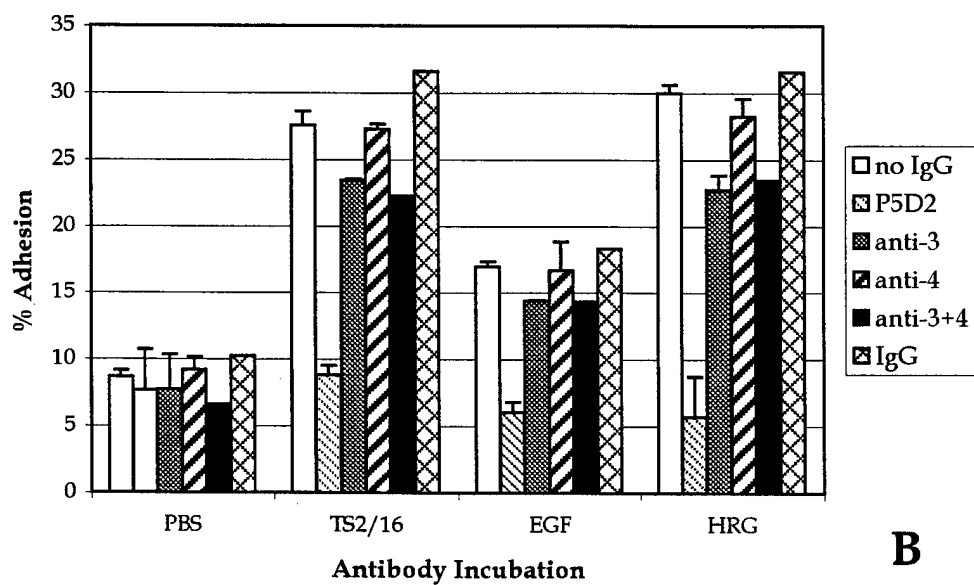


FIGURE 5

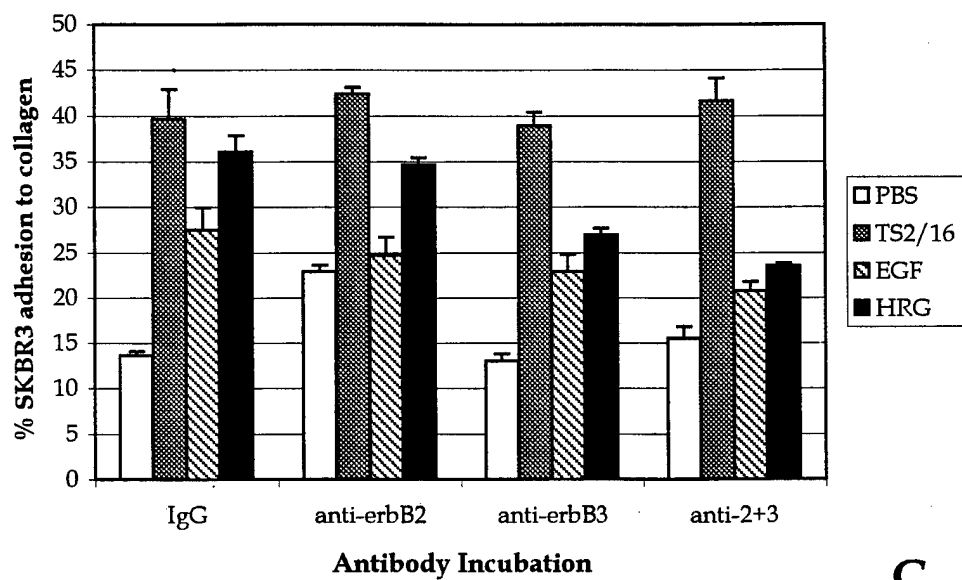


A

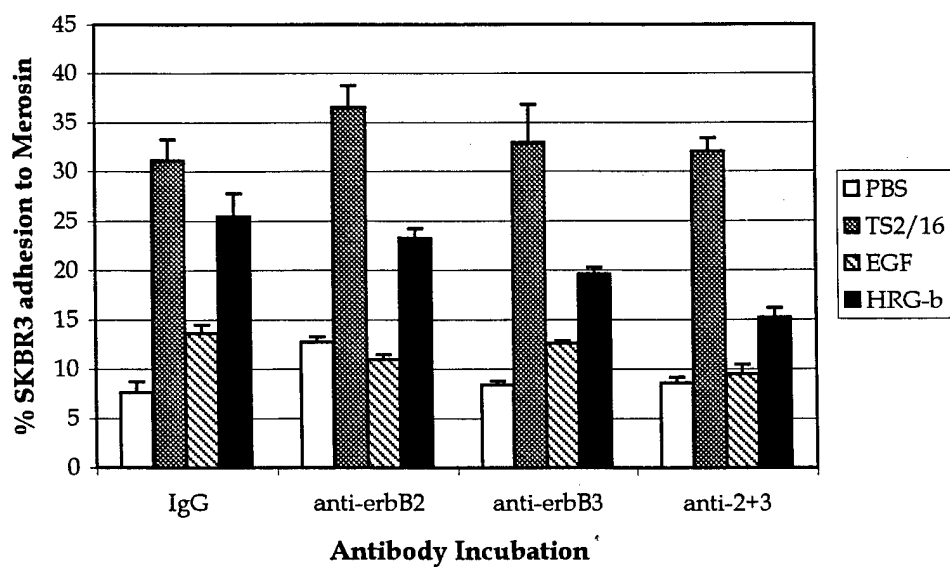


B

FIGURE 5



C



D

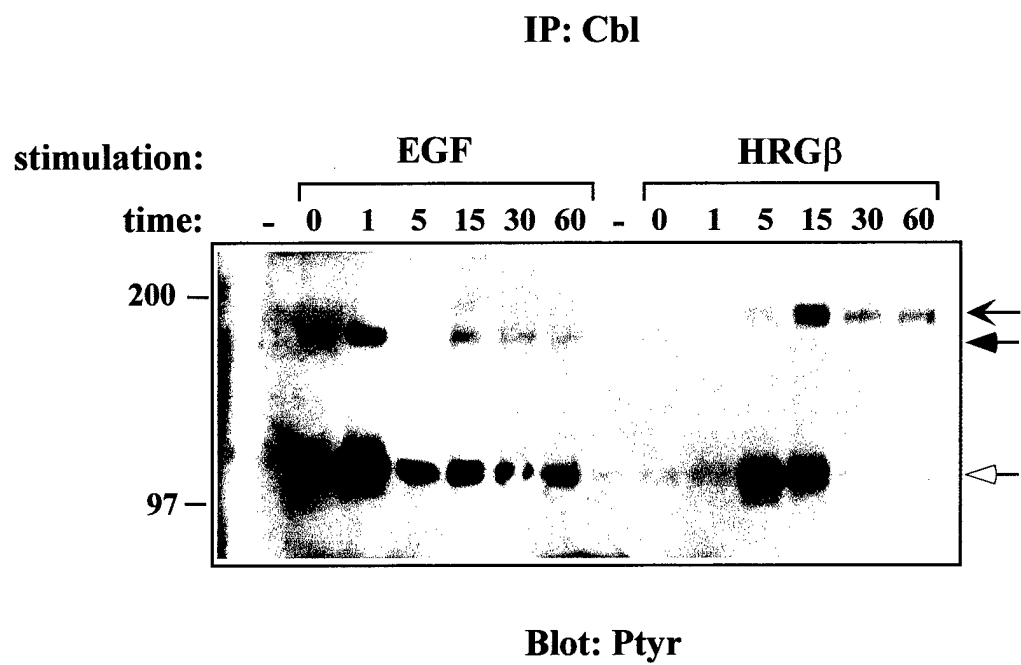


Figure 6

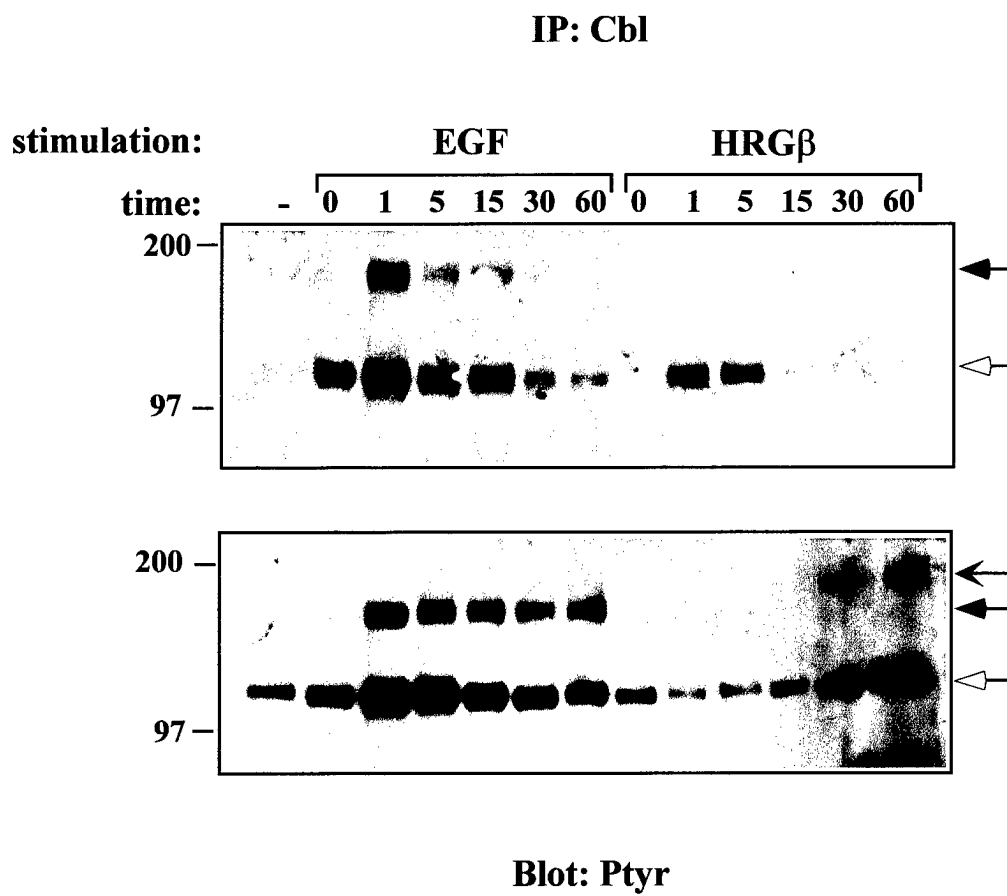
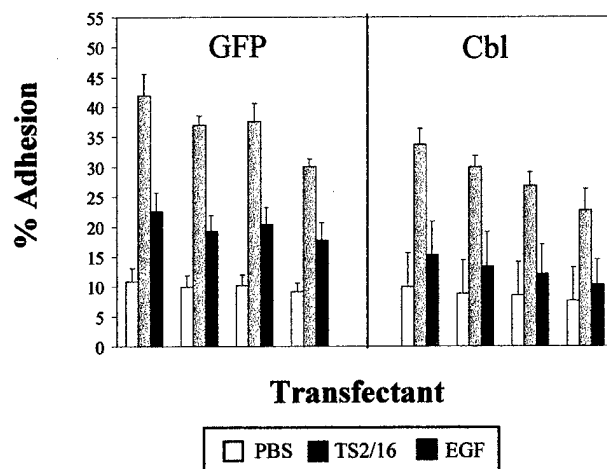


Figure 7

A



B

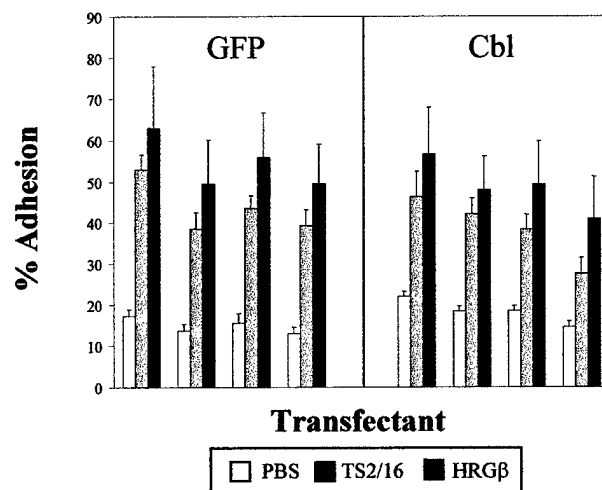
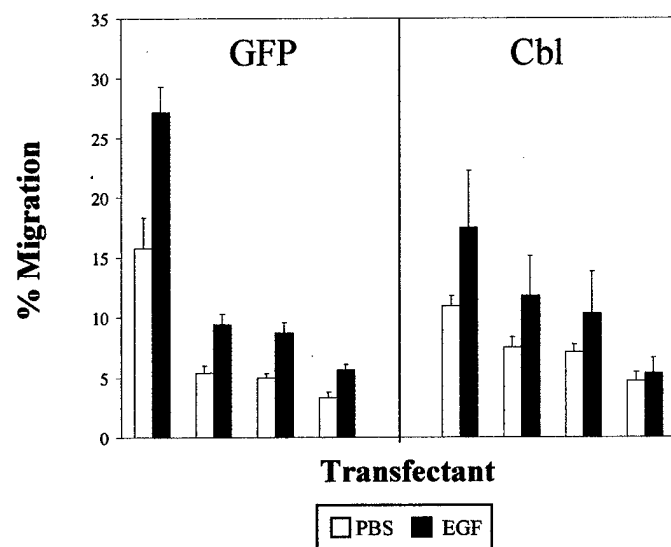


Figure 8

A



B

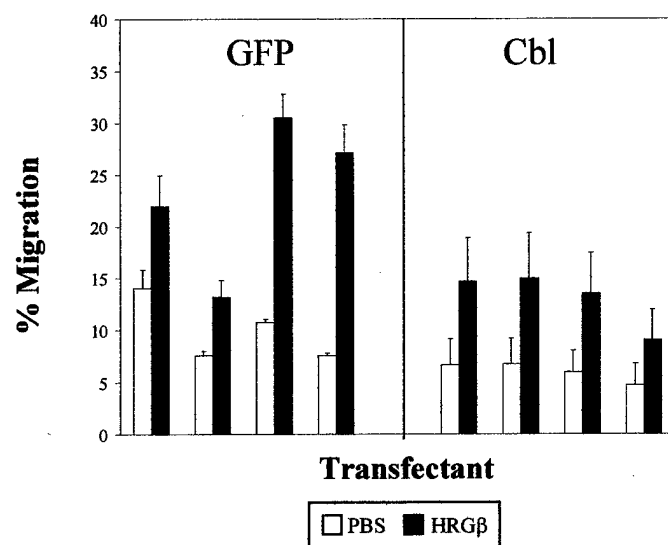
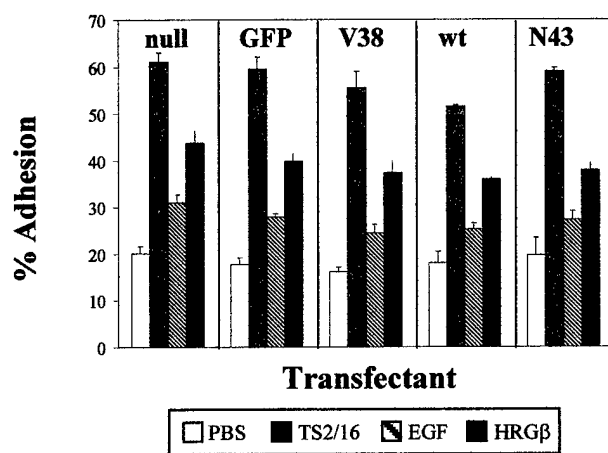


Figure 9

A



B

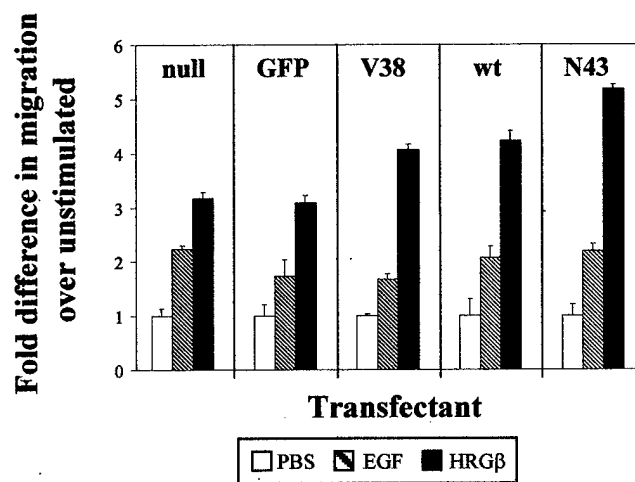


Figure 10

Stimulation of $\beta 1$ -Integrin Function by Epidermal Growth Factor and Heregulin- β Has Distinct Requirements for erbB2 but a Similar Dependence on Phosphoinositide 3-OH Kinase

Margaret A. Adelsman,^{*†‡} James B. McCarthy,^{*†} and Yoji Shimizu^{*†§}

^{*}Department of Laboratory Medicine and Pathology, [†]Center for Immunology, [‡]Cancer Center, University of Minnesota, Minneapolis, Minnesota 55455

Submitted February 19, 1999; Accepted July 12, 1999
Monitoring Editor: Carl-Henrik Heldin

Integrins and growth factor receptors are important participants in cellular adhesion and migration. The EGF receptor (EGFR) family of tyrosine kinases and the $\beta 1$ -integrin adhesion receptors are of particular interest, given the implication for their involvement in the initiation and progression of tumorigenesis. We used adhesion and chemotaxis assays to further elucidate the relationship between these two families of transmembrane signaling molecules. Specifically, we examined integrin-mediated adhesive and migratory characteristics of the metastatic breast carcinoma cell line MDA-MB-435 in response to stimulation with growth factors that bind to and activate the EGFR or erbB3 in these cells. Although ligand engagement of the EGFR stimulated modest $\beta 1$ -dependent increases in cell adhesion and motility, heregulin- β (HRG β) binding to the erbB3 receptor initiated rapid and potent induction of breast carcinoma cell adhesion and migration and required dimerization of erbB3 with erbB2. Pharmacologic inhibitors of phosphoinositide 3-OH kinase (PI 3-K) or transient expression of dominant negative forms of PI 3-K inhibited both EGF- and HRG β -mediated adhesion and potently blocked HRG β - and EGF-induced cell motility. Our results illustrate the critical role of PI 3-K activity in signaling pathways initiated by the EGFR or erbB3 to up-regulate $\beta 1$ -integrin function.

INTRODUCTION

The integrin family of adhesion receptors plays a pivotal role in a wide variety of events that control a cell's communication with its environment (Diamond and Springer, 1994; Schwartz *et al.*, 1995; Aplin *et al.*, 1998). These include such functions as cellular adhesion, motility, survival, differentiation, and morphogenesis. The EGF receptor (EGFR) family of growth factor receptors also contributes in diverse ways to these events, and the signaling cascades governing the cellular outcomes initiated by signals from either receptor family are now being elucidated. The observation that growth factor receptors and integrins synergistically potentiate given biochemical events illustrates the interplay between these two families of cell surface receptors (Miyamoto *et al.*, 1996; Schneller *et al.*, 1997; Guilherme *et al.*, 1998; Woodard *et al.*, 1998). To better understand the normal and

aberrant communications that may contribute to cellular dysregulation present in tumorigenic and metastatic cells, it is crucial to characterize the potential pathways linking growth factor receptors to integrins and the outcome of these signals on events such as cellular adhesion and metastasis.

The $\beta 1$ -integrin subfamily interacts with various cellular counter-receptors and extracellular matrix (ECM) components determined by the partnering of specific α subunits with $\beta 1$, as well as the particular cellular context. Alterations in levels of expression of $\beta 1$ -integrins have been implicated in tumorigenesis (Albelda, 1993), although few consistent models have emerged to clarify how these changes contribute to cellular dysregulation in tumor cells. Recent studies using targeted disruption of the $\beta 1$ -integrin gene have illustrated the contribution of $\beta 1$ -integrins to the metastatic potential of murine lymphoma cells in vivo (Stroeken *et al.*, 1998), and manipulation of $\beta 1$ function by transgenic expression of a chimeric $\beta 1$ molecule has further demonstrated the importance of $\beta 1$ -integrins in normal epithelial cell proliferation, apoptosis, differentiation, and maintenance of cell polarity in the developing mammary gland (Faraldo *et al.*, 1998). However, expression levels of integrins, per se, may

[§] Corresponding author. E-mail: shimi002@tc.umn.edu.

Abbreviations used: COLL, collagen; ECM, extracellular matrix; EGFR, EGF receptor; FACS, flow cytometric analysis; FN, fibronectin; GFP, green fluorescent protein; HRG, heregulin; LAM, laminin; PI 3-K, phosphoinositide 3-OH kinase.

not necessarily translate into a commensurate functional outcome (Akiyama *et al.*, 1990; Shimizu *et al.*, 1990a,b), because integrin function can be rapidly and transiently regulated in response to stimulation of several cell surface receptors. This fine-tuned control of $\beta 1$ -integrin function in the absence of alterations in integrin levels at the cell surface is elegantly demonstrated on circulating leukocytes (Shimizu, 1994), and rapid up-regulation of $\beta 1$ -integrins after stimulation of receptor tyrosine kinases such as the PDGF or c-kit receptors expressed on mast cells (Kinashi and Springer, 1994; Kinashi *et al.*, 1995; Serve *et al.*, 1995; Vosseller *et al.*, 1997) has also been described. However, the potential for signaling between the multisubunit EGFR family of receptors and the $\beta 1$ -integrins has not been extensively examined.

The EGFR family of receptor tyrosine kinases is now recognized as a multisubunit family consisting of the EGFR (erbB1), erbB2, the kinase-impaired erbB3, and erbB4. These receptors, with the exception of erbB2, are bound and activated by distinct sets of growth factors that fall broadly into three categories. The first includes EGF, amphiregulin, and TGF α , growth factors specific for the EGFR. Secondly, the heregulins (HRGs) represent a multigene family of growth factors with alternately spliced forms that bind specifically to erbB3 and erbB4. Finally, more promiscuous growth factors such as betacellulin, heparin-binding EGF, and epiregulin are capable of interacting with both the EGFR and with erbB4. Adding to the complexity of this family is the dramatic potential for signal diversity attributable to homo- and heterodimerization, as well as possible secondary dimerization (Graus-Porta *et al.*, 1997; Huang *et al.*, 1998) between its members. The formation of and unique biochemical properties of these ligand-driven heterodimers are now being appreciated (Lemmon and Schlessinger, 1994; Earp *et al.*, 1995; Wallasch *et al.*, 1995; Karunagaran *et al.*, 1996; Cohen *et al.*, 1996; Zhang *et al.*, 1996; Alroy and Yarden, 1997; Graus-Porta *et al.*, 1997; Riese and Stern, 1998). However, characterization of the unique biochemistry presented by specific heterodimers has just begun, and the possible contribution of different receptor combinations in cellular adhesion and motility has remained relatively unexplored. It is likely that the signaling pathways leading to mitogenesis are quite distinct from those of other cellular events such as motility or invasion (Chen *et al.*, 1994; Elenius *et al.*, 1997), and recent studies have made important contributions toward dissecting the EGF-sensitive motility responses (Chen *et al.*, 1996; Ware *et al.*, 1998; Xie *et al.*, 1998; Li *et al.*, 1999). Nonetheless, experimental systems used to assess EGF regulation of motility have often used exogenous expression of the EGFR in receptor-negative cells or cell lines with artificially high receptor levels. In addition, little experimental work has been directed at integrin-mediated events in response to HRG.

The rapid and transient up-regulation of $\beta 1$ function on leukocytes (Shimizu, 1994) provides a compelling parallel with metastatic processes undertaken by aggressive tumor cells, and the lipid kinase phosphoinositide 3-OH kinase (PI 3-K) has emerged as a critical component in many of the pathways that contribute to the regulation of $\beta 1$ -integrin function (Shimizu and Hunt 1996). Cell surface receptors, including the EGFR family, in a host of cell types and with varying functions interact directly or indirectly with PI 3-K and stimulate its enzymatic activity, thereby generating

lipid byproducts that are now believed to participate directly, both in a positive and negative manner, in pathways critical to mitogenesis, cell survival and apoptosis, adhesion, motility, and cytoskeletal reorganization (Kapeller and Cantley, 1994; Carpenter and Cantley, 1996; Klippel *et al.*, 1997; Stokoe *et al.*, 1997; Falasca *et al.*, 1998). Importantly, PI 3-K has emerged as a critical enzyme in the basal motility of other breast carcinoma cell lines (Keely *et al.*, 1997). In addition, activation of PI 3-K by the $\alpha 6 \beta 4$ -integrin has been implicated in the enhanced migration of a breast cancer cell line expressing transfected $\beta 4$ -integrin (Shaw *et al.*, 1997). Given these reports, it seems plausible to suggest that members of the EGFR family may facilitate $\beta 1$ -integrin-mediated adhesion and migration via activation of PI 3-K, particularly in tumor cell lines that have not been altered via transfection of integrin subunits or EGFR family members.

The role of PI 3-K in EGFR signaling and in the regulation of integrin function in the immune system suggests a potential synergy between EGFR signaling and integrin function in breast cancer. Therefore, we have dissected the contributions of members of the EGFR family of receptor tyrosine kinases to the regulation of $\beta 1$ -integrin function in breast cancer cells and examined the role of PI 3-K in these pathways. Our data demonstrate rapid up-regulation of $\beta 1$ -integrin function by ligand stimulation of the EGFR or erbB3, in a PI 3-K-dependent manner and illustrate the preferential participation of erbB2 in HRG β - rather than EGF-stimulated adhesion and migration.

MATERIALS AND METHODS

Cell Lines

The MDA-MB-435 cell line was maintained in Leibovitz's L-15 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (Atlanta Biologicals, Norcross, GA). The 528 hybridoma, expressing the anti-EGFR monoclonal antibody, was maintained in RPMI 1640 medium (Mediatech, Washington, DC) containing 10% FCS. Culture supernatant was harvested from confluent cultures of 528 cells and was titered for detection of the EGFR. All cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA), and all cell culture media contained additives of 2 mM L-glutamine and 50 U/ml penicillin/streptomycin (Mediatech).

Flow Cytometry

Single-color flow cytometric analysis (FACS) was performed on cells in suspension after removal from tissue culture flasks with EDTA or trypsin. Cells (5×10^5) were typically analyzed with antibodies incubated as 1 μ g purified antibody, 5 μ l ascites antibody, or 25 μ l antibody in culture supernatant/ 1×10^6 cells. Antibodies in the form of ascites or culture supernatant were routinely titered for appropriate detection of cell surface receptors. Antibodies for flow cytometric analysis included the anti-EGFR monoclonal antibody 528 (ATCC), the anti-erbB2 monoclonal Ab-5 (Calbiochem, La Jolla, CA), the anti-erbB3 monoclonal antibody Ab-4, the anti-erbB4 monoclonal antibody Ab-1 (Lab Vision, Fremont, CA), the $\beta 1$ -integrin-specific monoclonal antibody TS2/16 (ATCC), the $\beta 2$ -integrin-specific monoclonal antibody TS1/18 (ATCC), the $\alpha 1$ -integrin-specific monoclonal TS2/7 (ATCC), the $\alpha 2$ -integrin-specific monoclonal antibody P1E6 (Life Technologies), the $\alpha 3$ -integrin-specific monoclonal antibody P1B5 (Life Technologies), the $\alpha 4$ -integrin-specific monoclonal antibody NIH49d-1 (a kind gift from Dr. S. Shaw, National Institutes of Health), the $\alpha 5$ -integrin-specific monoclonal antibody P1D6 (Life Technologies), the $\alpha 6$ -integrin-specific monoclonal antibody GoH-3 (ICN/Cappell, Co-

chranville, PA), and FITC-conjugated goat anti-mouse IgG or goat anti-rat IgG (Southern Biotechnology, Alabaster, AL). Cells in FACS buffer (HBSS containing 1% bovine calf serum [Hyclone Laboratories, Logan, UT]) were incubated with appropriate antibodies for 30 min on ice, washed three times in FACS buffer, and incubated for an additional 30 min with appropriately diluted FITC-conjugated secondary antibodies. After two washes in ice-cold FACS buffer, data were acquired on a Becton Dickinson (Mountain View, CA) FACS-can or FACScalibur and analyzed using Cellquest software.

DNA Constructs and Transfections

The green fluorescent protein (GFP)-wild-type p85 and GFP- Δ p85 constructs have been previously described (Chan *et al.*, 1997). Transfections were carried out by electroporation in 4-mm gap cuvettes (Invitrogen, San Diego, CA). Cells (5×10^6) in 300 μ l Opti-MEM (Life Technologies) were incubated with 25 μ g appropriate DNA and electroporated using 250-V, 960- μ F settings on a Bio-Rad (Hercules, CA) gene pulser with capacitance extension. After allowing the cells to recover for 20 min at room temperature, cells were transferred to tissue culture flasks containing 20% FCS and 80% L-15 media and were allowed to recover for 24–48 h before use in adhesion or migration assays. Typical transient expression of DNA constructs ranged from 15–35% of recovered cells.

Adhesion Assays

Standard adhesion assays were performed using cells labeled with Calcein AM (Molecular Probes, Eugene, OR) as previously described (Zell *et al.*, 1996). ECM ligands were human type IV collagen (Sigma, St. Louis, MO), mouse Engelbreth-Holm-Swarm Sarcoma (EHS)-derived type IV collagen (Life Technologies), human merosin or EHS-derived laminin (Life Technologies), and human fibronectin (FN). For transient expression of GFP fusion proteins, adhesion was quantitated after collection of adherent cells and analysis by flow cytometry essentially as described (Chan *et al.*, 1997; Kivens and Shimizu, 1999). Growth factor stimulation was performed with EGF (Life Technologies), betacellulin, HRG α , or HRG β (all from R&D Systems, Minneapolis, MN). For receptor blocking studies, cells were incubated in the presence of control mouse IgG (Caltag, South San Francisco, CA), the anti- $\beta 1$ -integrin antibody P5D2 (a kind gift from T. LeBien, University of Minnesota, Minneapolis, MN), the anti-erbB2 Ab-16, the anti-erbB3 Ab-5, or the anti-erbB4 Ab-3 (all from Lab Vision) at 1 μ g antibody/ 1×10^6 cells or as indicated in figure legends. Tyrophostin AG1478 (Calbiochem) was used for inhibition of the EGFR. Pharmacological inhibition of PI 3-K was performed with wortmannin (Sigma) or LY294002 (Alexis, San Diego, CA). Inhibition of mitogen-activated, ERK-activating kinase was performed using the inhibitor PD98059 (Parke-Davis, Ann Arbor, MI).

Migration Assays

Cell lines were allowed to grow to confluency (~75–85%) before harvest for migration studies. Subconfluent cell cultures were placed in serum-free media for 12–24 h and harvested by releasing from flasks with 1 mM EDTA. After cells were washed free of EDTA in serum-free RPMI 1640 media, they were quantitated and assessed for viability using trypan blue. Cells at a density of 400,000 cells/ml in assay media (RPMI, 20 mM HEPES, 0.1% BSA) were added in 57 μ l to the upper well of a 48-well chemotaxis chamber (Neuroprobe, Cabin John, MD), containing assay media or appropriate growth factor. Polycarbonate filters (8 μ m; Osmonics, Livermore, CA) were precoated with mouse EHS-derived type IV collagen or EHS-derived laminin (Life Technologies) at 20 μ g/ml in PBS overnight at 4°C and allowed to air dry before placing in chambers. Cells were allowed to migrate in the presence or absence of stimulators for 4–6 h at 37°C before disassembly of the chambers, fixing, and staining of the migrated cells. Nonmigrated cells were removed from the upper

surface of the filters after placing on a microscope slide, and cell migration was quantitated by counting and taking the sum of migrated cells in four separate fields of at least three individual wells. For inhibition studies, cells were preincubated for 15 min on ice with inhibitor or appropriate control before addition to chemotaxis chambers. Although some variability in basal cell migration was observed in separate experiments, relative changes between stimulated and unstimulated migration were consistent.

For transient transfection-migration assays cells were transfected as described above. Cells were serum starved for 12 h before harvesting for migration assays. Transwell chambers (six-well size, 8- μ m filters; Costar, Cambridge, MA) or 8- μ m polycarbonate membrane filters for Boyden chemotaxis chambers were coated overnight at 4°C in solutions of mouse EHS-laminin or EHS-collagen at 20 μ g/ml in PBS. Growth factors diluted in assay media were added to the lower wells of chemotaxis chambers or transwells, and coated filters were placed on top. Cells were then added to upper wells at $\sim 1 \times 10^6$ cells per well in 1.5 ml assay media. The same dilution of cells was used for addition to quadruplicate wells in 24-well plates (100 μ l/well) for determination of starting cell populations. Cells were also added to the upper wells of Boyden chambers, and migration was allowed to proceed overnight in both transwells and Boyden chambers. Chemotaxis chambers were disassembled and analyzed the following morning as described above. Transwell migration chambers were disassembled, and migrated cells were removed from the lower surface of each well with 1:1 trypsin:EDTA. Dislodged cells were added to FACS tubes containing ice-cold 10% FACS buffer (HBSS and 10% bovine calf serum), spun, and resuspended in 200 μ l 10% FACS buffer. Cells plated in 24-well plates were also harvested and placed into FACS tubes for approximation of cells added per well and for determining the percent efficiency of transfectants. FACS tubes containing representative starting cell populations or migrated cell populations were analyzed by flow cytometry with additions of 25,000 reference beads per tube (9.7 μ m; Interfacial Dynamics, Portland, OR) to determine cell numbers present in each tube. Quantitation was done essentially as described (Chan *et al.*, 1997). Briefly, the numbers of cell and reference bead events acquired by flow cytometric analysis were used to calculate cell numbers present in starting populations composed of GFP $^-$, GFP $^+$, GFP $^{++}$, and GFP $^{+++}$ (fluorescing between logs 0 and 1, 1 and 2, 2 and 3, or 3 and 4, respectively). Using reference beads, the number of cells in each migrated population was determined in the same way and compared directly with the starting cell population to calculate percent migration and fold change. Comparison of assays carried out for 4 versus 16 h gave similar results. The average of three wells per condition was determined for each data point (Kivens and Shimizu, 1999).

Immunoprecipitation

Cells that had been serum starved for 12–24 h were harvested from tissue culture flasks using 1 mM EDTA. Cells were washed in serum-free RPMI 1640 medium to remove EDTA and were quantitated by trypan blue exclusion. Equal aliquots of cells were added to Eppendorf tubes and stimulated in the presence or absence of growth factors for the indicated periods at 37°C. For studies assessing the effects of erbB2 blocking, cells were preincubated on ice for 15 min with mouse IgG as control or erbB2 blocking Ab-16 at 0.5 μ g/ 1×10^6 cells before stimulation. After stimulation, cells were lysed directly in 0.5 ml 2 \times lysis buffer (1 \times = 1% Triton X-100, 1% deoxycholic acid, 158 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.2, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM sodium orthovanadate) on ice for 20 min. Supernatants were clarified by centrifugation for 20 min at 4°C, and postnuclear supernatants were immunoprecipitated with the anti-phosphotyrosine antibody PY20 (10 μ l 1:10 ascites, provided by Dr. M. Kamps, University of California, San Diego, CA), the EGFR mAb 528 (15 μ l culture supernatant), the erbB2 mAb-5 (1 μ g), or the erbB3 mAb4 (1 μ g) overnight at 4°C. Protein-A Sepharose 4B or goat anti-mouse

IgG-Sepharose 4B (Zymed, San Francisco, CA; 50 μ l/tube) was added the following morning for an additional 1-h incubation, and immunocomplexes were washed twice in 1 \times lysis buffer containing protease inhibitors. Protein A-Sepharose or goat anti-mouse Sepharose-bound proteins were boiled for 4 min in the presence of 2 \times SDS-sample buffer (125 mM Tris, pH 6.8, 4% SDS, 2 mM EDTA, 20% glycerol, 10% β -mercaptoethanol, 0.6% bromophenol blue) and were separated on 10% polyacrylamide gels by SDS-PAGE.

Western Blotting

Cell lysates or immunoprecipitates were separated by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Bedford, MA) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.075% SDS) for 2 h at 400 mA. Membranes were incubated in blocking buffer (5% Carnation milk and PBS) for 1 h at room temperature or overnight at 4°C. Blots were rinsed in PBS before addition of primary antibodies diluted in blocking buffer (4G10, Upstate Biotechnology, Lake Placid, NY; anti-EGFR sc-03, Santa Cruz Biotechnology, Santa Cruz, CA; anti-p85, Upstate Biotechnology; anti-erbB3 Ab-7, Lab Vision; anti-erbB2 Ab-10, Lab Vision) for 1 h at room temperature. Blots were rinsed three times in PBS and 0.1% Tween 20 for 10 min each before addition of secondary antibodies diluted in blocking buffer (goat anti-mouse IgG-HRP; Life Technologies) or donkey anti-rabbit-IgG-HRP (Amersham, Arlington Heights, IL) for 1 h at room temperature. Blots were rinsed three times in PBS and 0.1% Tween 20, and bands were visualized using enhanced chemiluminescence (Pierce, Rockford, IL). For reprobing membranes, stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, 0.1 M β -mercaptoethanol) was used at 50°C for 30 min followed by blocking membranes in 5% milk and PBS and reprobing with appropriate antibodies.

In Vitro PI 3-Kinase Assays

PI 3-Kinase assays were performed with PY20 immunoprecipitates of 10×10^6 cells per sample as previously described (Chan *et al.*, 1997). Phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL) was used as a substrate for PY20-associated PI 3-kinase, and radioactive lipid products were separated by TLC and visualized by autoradiography. Quantification was performed by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA) of the TLC plates. Similar results were obtained from a minimum of three independent assays.

RESULTS

Adhesion of MDA-MB-435 Breast Carcinoma Cell Lines to β 1-Integrin Ligands Can Be Regulated by Stimulation of EGFR Family Members

To test our hypothesis that stimulation of EGFR family receptors might up-regulate β 1-integrin function in breast cancer cells, we determined the expression of these receptor tyrosine kinases on the surface of metastatic MDA-MB-435 cells and examined their ability to respond to growth factor stimulation by up-regulating adhesion to β 1-integrin ECM ligands. MDA-MB-435 cells express moderate levels of the EGFR, erbB2, and erbB3 (Figure 1A), with no detectable erbB4 protein by flow cytometry (Figure 1A) or blotting methods (our unpublished results). Because erbB2 is still considered an "orphan" receptor in that no growth factor has yet been found by which erbB2 is directly bound and activated, the presence of both EGFR and erbB3 on the surface of these cells led us to investigate growth factors that specifically bind to and activate either the EGFR or erbB3. These cells exhibited inducible adhesion to several β 1-integrin

ligands such as FN, EHS-derived laminin (LAM), or merosin (our unpublished data), but the strongest inducible adhesion was consistently observed on type IV collagen (COLL) in response to EGF or HRG β (Figure 1B). HRG β -induced adhesion was typically 1.5- to 2-fold higher than that induced by EGF and was slightly less than adhesion achieved by directly activating the β 1-integrin with the monoclonal antibody TS2/16 (Arroyo *et al.*, 1992; Kovach *et al.*, 1992). β 1-integrins were the major adhesion receptors responsible for this event, as indicated by the nearly complete inhibition of both unstimulated and stimulated (TS2/16, EGF, or HRG β) adhesion to COLL by these cells when a blocking antibody against β 1 was used (Figure 1C). EGF-stimulated adhesion to COLL was induced in a rapid manner, with maximal increases over unstimulated adhesion found by 10–20 min of stimulation at 37°C (Figure 1D). Time courses of inducible adhesion to other ECM ligands examined were similar, and the maximal time of stimulated adhesion for HRG β was similar to that of EGF (our unpublished data). Thus, adhesion assays were typically performed for 10 min at 37°C.

To further evaluate the stimulation of β 1-integrin adhesion by EGFR family receptors, the dose-response curves for several growth factors capable of interacting with EGFR or erbB3 were assessed. EGF- and HRG β -mediated adhesion to COLL were dose dependent, with maximal stimulation of adhesion peaking at 10 ng/ml EGF (Figure 2A) or 100 ng/ml HRG β (Figure 2B), with a plateau of maximal adhesion at higher concentrations of growth factor. Based on these findings, we typically stimulated cells with 100 ng/ml EGF or HRG β in cellular adhesion assays. Betacellulin stimulation of the EGFR also increased MDA-MB-435 cell adhesion to COLL in a dose-dependent manner, with maximal stimulation of adhesion paralleling that found with EGF stimulation in the same assay (Figure 2C). Examination of HRG α , a growth factor that binds only to erbB3 or erbB4, showed no effects on adhesion of this cell line at any of the concentrations of growth factor tested (Figure 2D).

To understand the receptor biochemistry in MDA-MB-435 cells in response to these various stimuli, we analyzed receptor immunoprecipitates of the EGFR (Figure 3A), erbB2 (Figure 3B), or erbB3 (Figure 3C) after stimulation with each of the four growth factors examined in Figure 2. EGF and betacellulin stimulated intense tyrosine phosphorylation of the EGFR and erbB2 with rapid and transient kinetics. HRG β strongly activated both erbB3 and erbB2 in a very sustained manner over the stimulation time course assessed. In contrast, HRG α had little effect on any of the EGFR family receptors in these cells, in keeping with the lack of adhesion response observed in Figure 2D. Thus, all three ligands active in these cells caused stimulation of their primary receptors, the EGFR for EGF and betacellulin and erbB3 for HRG β , as well as phosphorylation of erbB2. These data suggest that stimulation of the EGFR with ligands such as EGF or betacellulin activates EGFR and erbB2 phosphorylation and significantly increases β 1-integrin-dependent adhesion of the MDA-MB-435 cell line to type IV collagen. Although erbB3 levels were not significantly higher than EGFR on these cells (Figure 1A), HRG β stimulation gave a much more potent induction of both erbB2 phosphorylation and β 1-mediated adhesion, suggesting a qualitative difference in the pathways used by these receptors. Importantly, this

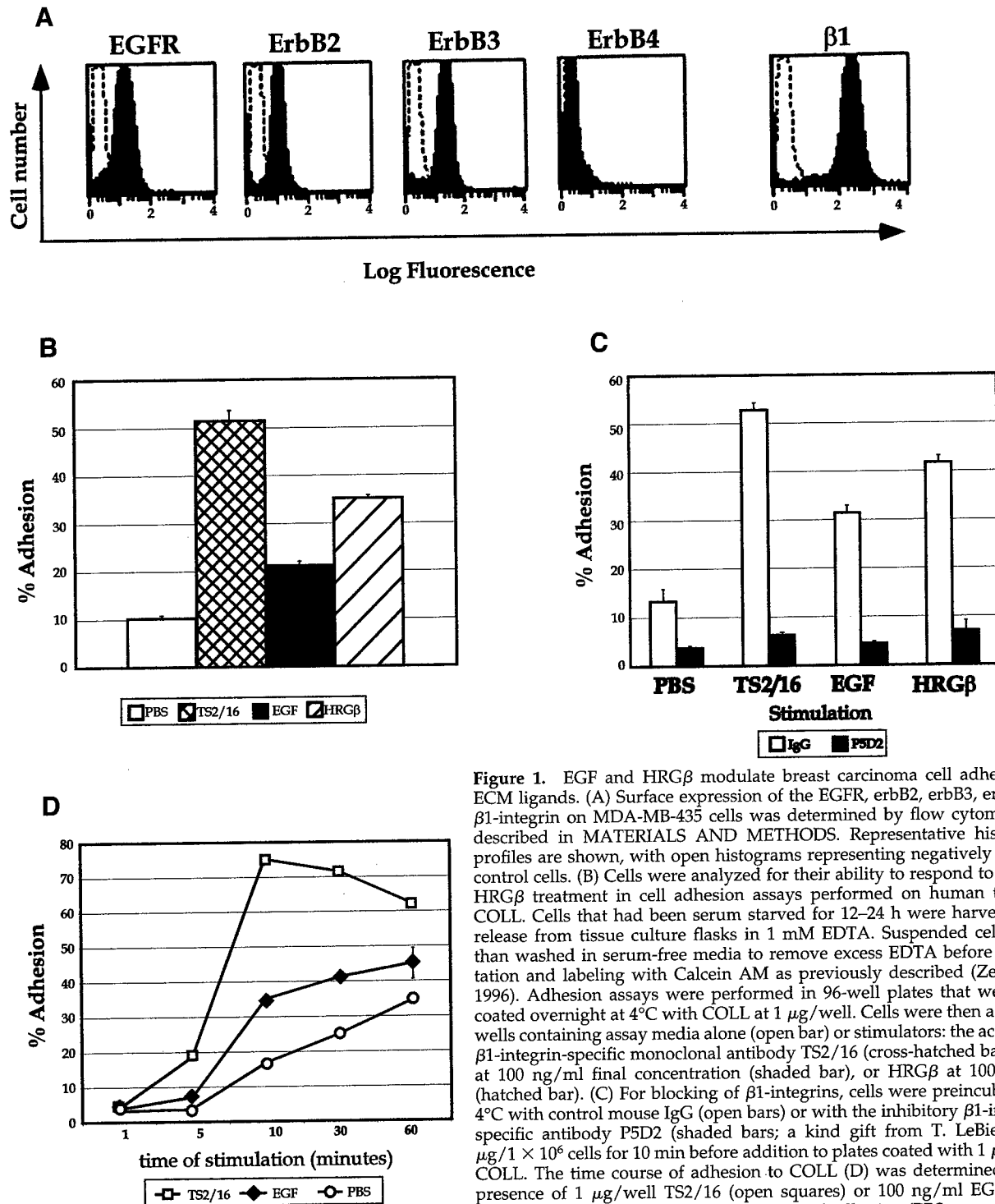


Figure 1. EGF and HRG β modulate breast carcinoma cell adhesion to ECM ligands. (A) Surface expression of the EGFR, erbB2, erbB3, erbB4, or $\beta 1$ -integrin on MDA-MB-435 cells was determined by flow cytometry as described in MATERIALS AND METHODS. Representative histogram profiles are shown, with open histograms representing negatively stained control cells. (B) Cells were analyzed for their ability to respond to EGF or HRG β treatment in cell adhesion assays performed on human type IV COLL. Cells that had been serum starved for 12–24 h were harvested by release from tissue culture flasks in 1 mM EDTA. Suspended cells were then washed in serum-free media to remove excess EDTA before quantitation and labeling with Calcein AM as previously described (Zell *et al.*, 1996). Adhesion assays were performed in 96-well plates that were pre-coated overnight at 4°C with COLL at 1 μ g/well. Cells were then added to wells containing assay media alone (open bar) or stimulators: the activating $\beta 1$ -integrin-specific monoclonal antibody TS2/16 (cross-hatched bar), EGF at 100 ng/ml final concentration (shaded bar), or HRG β at 100 ng/ml (hatched bar). (C) For blocking of $\beta 1$ -integrins, cells were preincubated at 4°C with control mouse IgG (open bars) or with the inhibitory $\beta 1$ -integrin-specific antibody P5D2 (shaded bars; a kind gift from T. LeBien) at 1 μ g/ 1×10^6 cells for 10 min before addition to plates coated with 1 μ g/well COLL. The time course of adhesion to COLL (D) was determined in the presence of 1 μ g/well TS2/16 (open squares) or 100 ng/ml EGF (solid diamonds) in comparison with unstimulated adhesion (PBS; open circles).

Cells were allowed to settle briefly in wells before analyzing preadherent fluorescence on a fluorescence plate reader. Cells were then stimulated at 37°C for 10 min or for designated times before removing nonadherent cells by hand washing with a syringe-manifold system. Comparison of adhesion with human type IV collagen or mouse EHS-collagen did not show significant differences (our unpublished data). Adhesion data are representative of at least three independent experiments and reflect the average of triplicate samples per condition.

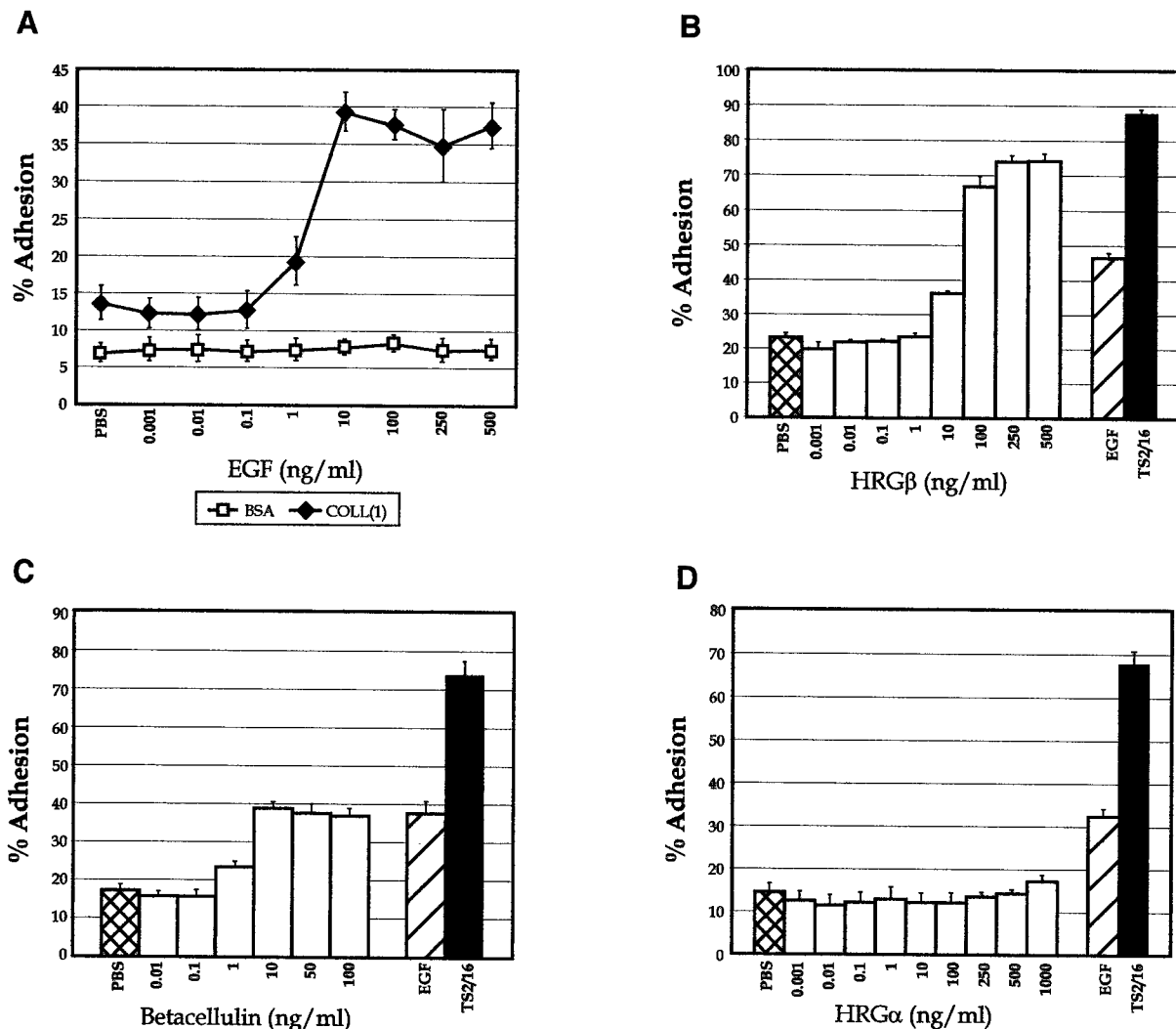


Figure 2. MDA-MB-435 cell adhesion is stimulated by multiple growth factors that bind to and activate members of the EGF receptor family. For analysis of dose response to growth factor, increasing amounts of EGF ranging from 1 pg/ml to 500 ng/ml were added to COLL-coated wells before addition of cells and stimulation at 37°C (A; solid diamonds). Adhesion to BSA-coated wells (open squares) was performed as a control. Dose responses for adhesion to COLL were determined in the presence of increasing amounts of HRGβ (B), the EGF-like growth factor betacellulin (C), or HRGα (D). Unstimulated (PBS; B–D, cross-hatched bars), EGF-stimulated (B–D, solid bars) adhesion was analyzed for comparison. Cells plated on BSA alone as a control for nonspecific adhesion generally showed <10% adhesion (A; our unpublished data). Data are representative of at least three separate assays.

up-regulation occurred in the absence of significant changes in either integrin subunit or EGFR family receptor numbers on the cell surface as assessed by flow cytometry (our unpublished data).

Stimulation of EGFR Family Members Increases Migration of MDA-MB-435 Breast Carcinoma Cells

Because the MDA-MB-435 cells are highly metastatic in nude mouse models (Price *et al.*, 1990), we also examined cellular migration and motility in vitro. Previous studies have described migration and adhesion of unstimulated MDA-MB-435 cells on COLL and LAM (Shaw *et al.*, 1996), and our FACS analyses showed strong expression of β 1-in-

tegrins capable of binding COLL and LAM (α 2, α 3, and α 6; our unpublished data), consistent with this earlier study. Thus, we examined the migration of unstimulated and stimulated MDA-MB-435 cells on both COLL and LAM. As found in our adhesion experiments, betacellulin, EGF, and HRGβ all increased the migration of 435 cells toward LAM (Figure 4A) in a dose-dependent manner, whereas parallel assays showed similar results on COLL (our unpublished data). Although the adhesion experiments showed increasing growth factor-stimulated adhesion with a plateau response (Figure 2), betacellulin and EGF both demonstrated bell-shaped curves for stimulated migration, with maximal responses at 0.1–1 and 1–10 ng/ml for betacellulin and EGF,

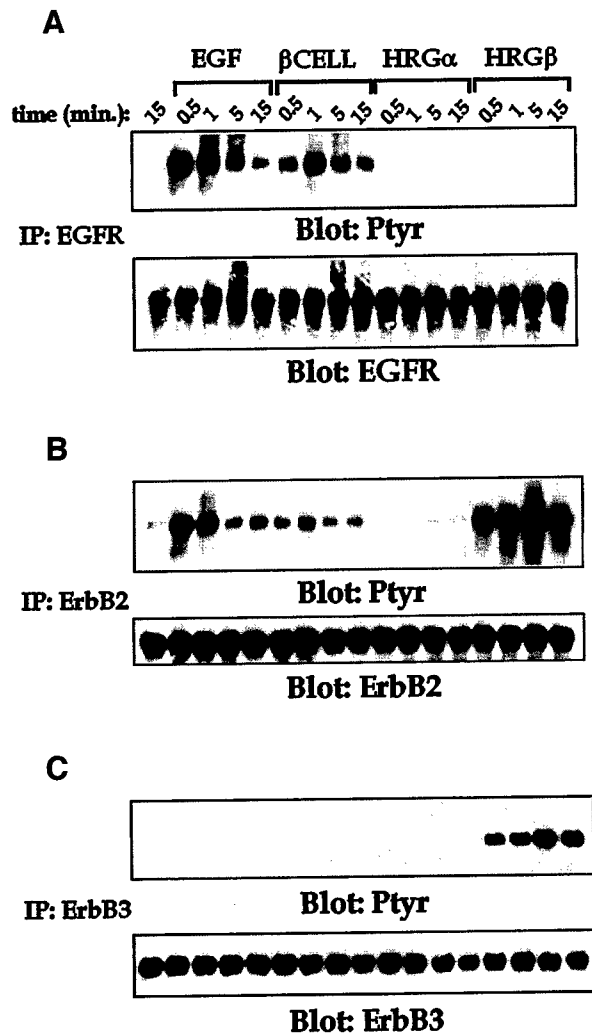


Figure 3. Multiple growth factors induce tyrosine phosphorylation of the EGFR, erbB2, and erbB3 in MDA-MB-435 cells. Receptor activation was assessed after stimulation of MDA-MB-435 cells (10×10^6 cells per sample) for increasing periods at 37°C with PBS alone (first lane in each panel), EGF at 100 ng/ml, betacellulin (βCELL) at 10 ng/ml, HRG α at 100 ng/ml, or HRG β at 100 ng/ml. Lysis buffer ($2\times$) was added to each sample after the indicated stimulation time, and cleared lysates were immunoprecipitated for EGFR (A), erbB2 (B), or erbB3 (C). Western blotting was performed on samples after separation by SDS-PAGE and transfer to polyvinylidene difluoride membranes. Total phosphotyrosine content in each sample was assessed by probing with anti-phosphotyrosine Ab 4G10 (upper panels). Equivalent receptor loading was confirmed by stripping and reprobing each blot for the presence of the indicated receptor (lower panels). Blots were also stripped and reprobed for the presence of p85 (our unpublished data). Similar results were observed in at least three independent experiments.

respectively (Figure 4A). Migration in response to HRG β showed similar dose effects as seen for adhesion, with strong induction of migration, reaching a maximal response by 100–250 ng/ml growth factor. The stimulated migration by HRG β was consistently much higher than that mediated by EGF, and both events were $\beta 1$ -integrin dependent as illus-

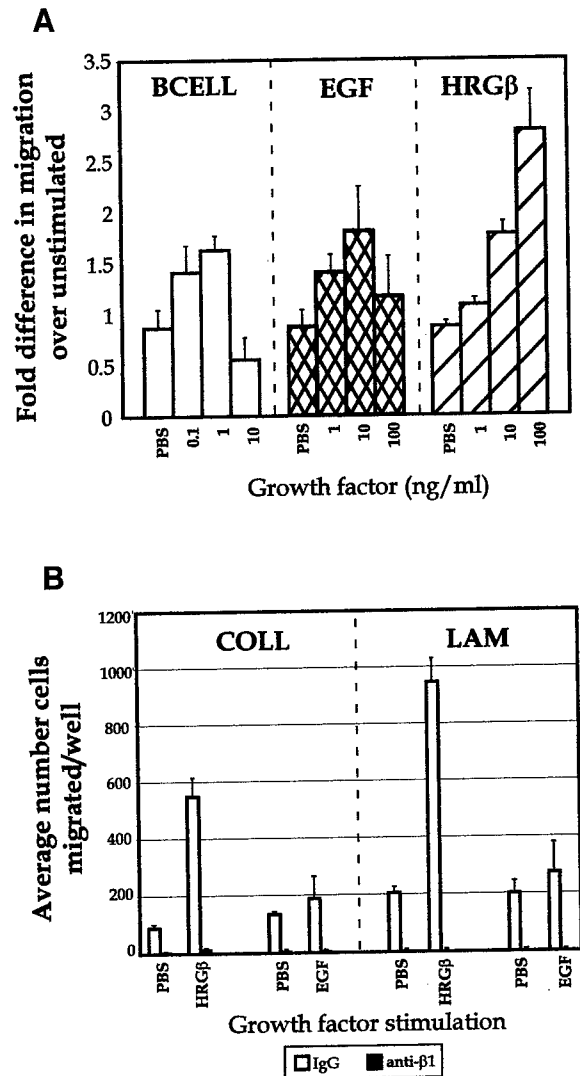


Figure 4. Betacellulin, EGF, and HRG β induce $\beta 1$ -integrin-dependent MDA-MB-435 cell migration on LAM. (A) Cells were grown to $\sim 75\%$ confluency and placed in serum-free media for 16 h before harvesting as for adhesion assays. Polycarbonate filters ($8 \mu\text{m}$) were coated overnight at 4°C in PBS containing mouse EHS-LAM or EHS-COLL (our unpublished data) at $20 \mu\text{g/ml}$. Forty-eight-well chemotaxis chambers were assembled with assay media alone or containing increasing amounts of growth factors in the lower chambers, as indicated. LAM-coated filters were placed over the lower wells and, $\sim 23,000$ MDA-MB-435 cells were placed in the upper wells and allowed to migrate at 37°C for 4–6 h. Migrated cells in each well were quantitated on fixed and stained filters. The sum of four microscopic fields was taken for each well, and three wells were averaged for each stimulation condition. (B) Antibody blocking studies were carried out by preincubating cells with control IgG (open bars) or the inhibitory $\beta 1$ -integrin-specific mAb P5D2 (solid bars) at $1 \mu\text{g}/1 \times 10^6$ cells on ice for 10 min before adding the cells to the upper wells of chambers containing EGF or HRG β and containing filters coated with LAM or COLL. Some variability was observed with the levels of basal migration in the data shown in A, because each growth factor titration was carried out in a separate chemotaxis chamber. However, the level of stimulation over that of basal migration for each growth factor was reproducible over at least three separate assays.

trated by the ability of an inhibitory $\beta 1$ -integrin-specific antibody to completely abrogate both unstimulated and EGF- or HRG β -stimulated migration toward COLL or LAM (Figure 4B). Thus, the adhesion and migration data support our hypothesis that growth factor stimulation of the EGFR couples to $\beta 1$ -integrin-mediated functional events. Furthermore, we have found that erbB3, a kinase-impaired receptor in the EGFR family, mediates potent stimulation of both adhesion and cell migration by the growth factor HRG β .

Contribution of Dimerization Partners with the EGFR and erbB3 in EGF and HRG β Regulation of $\beta 1$ -Integrins

The observation that two mechanistically distinct growth factors, EGF and HRG β , were capable of activating erbB2 phosphorylation and stimulating $\beta 1$ -integrin activity in MDA-MB-435 cells, coupled with the complex heterodimerization potential of the EGFR family of receptors (Lemmon and Schlessinger, 1994; Earp *et al.*, 1995; Riese and Stern, 1998), led us to investigate the potential contribution of erbB2 as a dimerization partner with the EGFR or with erbB3 in mediating the unique effects of EGF and HRG β on $\beta 1$ -integrin function. An anti-erbB3 antibody that blocks HRG β binding (Chen *et al.*, 1996) specifically abrogated HRG β -induced adhesion of MDA-MB-435 cells without affecting EGF- or TS2/16-stimulated adhesion, even at high concentrations of antibody (Figure 5A and our unpublished data). Additionally, an anti-erbB2 antibody that blocks the effects of EGF or HRG β binding to the dimerization partners of erbB2 (Klapper *et al.*, 1998) negated HRG β -stimulated adhesion without specifically affecting EGF or TS2/16 stimulation conditions (Figure 5A). The combination of both anti-erbB3 and anti-erbB2 antibodies gave a slightly stronger reduction in adhesion, but this decrease was consistent across unstimulated and all stimulated adhesion conditions. Although we could not detect erbB4 protein expression in our experiments with the MDA-MB-435 cells, it was possible that low but undetectable levels of erbB4 might be mediating the HRG β effects that we observed. However, an antibody that blocks HRG β binding to erbB4 (Chen *et al.*, 1996) did not inhibit HRG β -induced adhesion, even when combined with the erbB3 blocking antibody (Figure 5B). Although no specific effects of the erbB2 or erbB3 blocking antibodies were seen on EGF-mediated adhesion, only EGF-mediated adhesion was abrogated by the highly EGFR-specific inhibitor tyrphostin AG1478 (Fry *et al.*, 1994) in a dose-dependent manner (our unpublished data). Because of the strong stimulation of cell migration initiated by HRG β , we extended our antibody blocking studies to COLL and LAM migration assays to determine the receptor subunits contributing to these signals. Similar to the adhesion assays, anti-erbB3 and anti-erbB2 antibodies blocked HRG β -stimulated MDA-MB-435 cell migration toward LAM (Figure 6, A and B) or COLL (our unpublished data). In addition, incubation of cells with the AG1478 tyrphostin had negligible effects on either unstimulated or HRG β -stimulated cell migration (our unpublished data). EGF-stimulated migration of MDA-MB-435 cells was not affected by incubation with the erbB2 inhibitory antibody, as had been observed in the adhesion assays (Figure 6C).

To further explore the lack of effect of the erbB2 blocking Ab-16 on EGF-stimulated adhesion and migration, we ana-

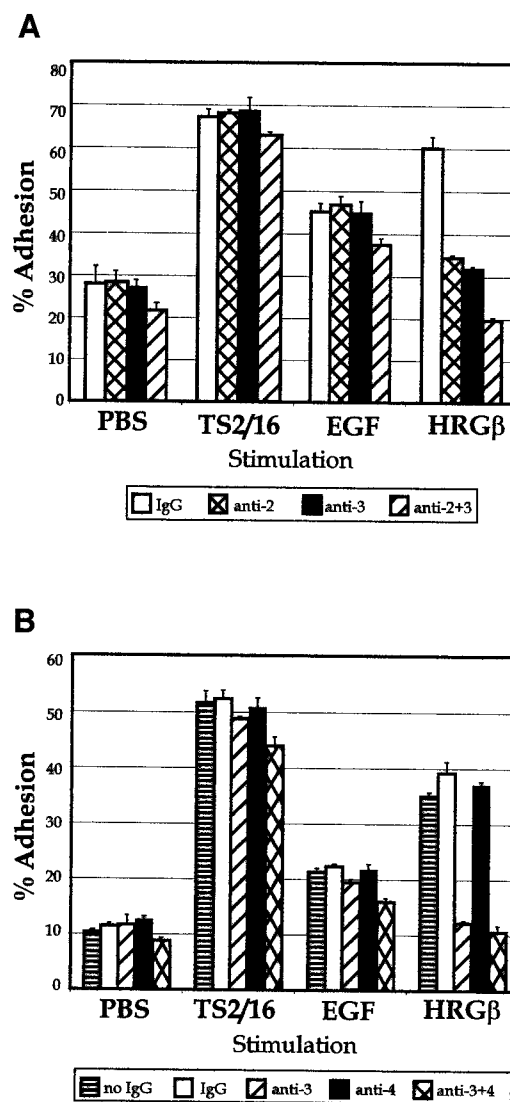


Figure 5. HRG β -mediated adhesion of MDA-MB-435 cells to COLL requires heterodimerization with erbB2. Adhesion assays were performed as described for Figure 1. (A) To examine contributions by erbB2 and erbB3, control IgG (open bars) or blocking mAbs specific for erbB2 (cross-hatched bars), erbB3 (shaded bars), or a combination of anti-erbB2 and anti-erbB3 mAbs (hatched bars) were incubated with cells before addition to plates containing TS2/16, EGF, or HRG β . (B) To examine contributions by erbB3 and erbB4, cells were incubated with no IgG (horizontally striped bars), control IgG (open bars), or blocking mAbs specific for erbB3 (hatched bars), erbB4 (solid bars), or a combination of anti-erbB3 and anti-erbB4 mAbs (cross-hatched bars) before addition to plates containing TS2/16, EGF, or HRG β . The data reflect an average of three wells per condition and are representative of at least three separate assays.

lyzed the phosphorylation status of erbB2 in response to EGF or HRG β after exposure to Ab-16. As previously demonstrated in Figure 3, both EGF and HRG β stimulate a time-dependent increase in the tyrosine phosphorylation of erbB2 (Figure 7), with EGF eliciting rapid and transient

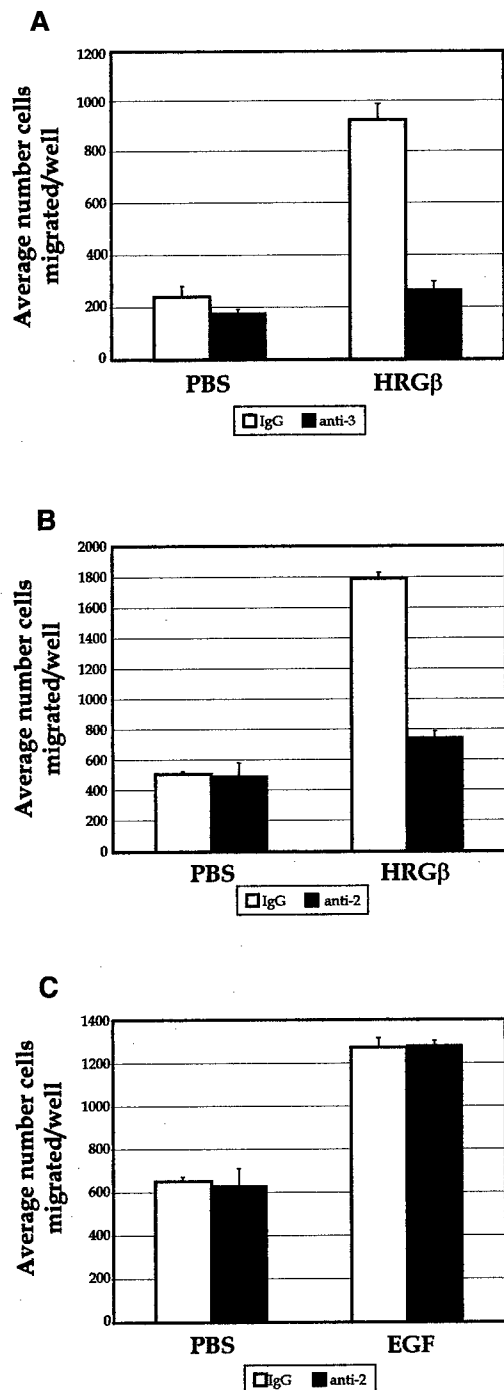


Figure 6. HRG β -mediated migration of MDA-MB-435 cells on LAM requires heterodimerization with erbB2. Migration assays were carried out as described for Figure 4. To examine contributions by erbB3 (A) or erbB2 (B and C), control IgG (open bars) or blocking mAbs specific for erbB3 (A, solid bars) or erbB2 (B and C, solid bars) were incubated with cells before addition to chemotaxis chambers containing control media, EGF, or HRG β . Cell migration was quantitated by taking the sum of four microscopic fields per well and the average of at least three wells per condition. Experiments were performed a minimum of three times.

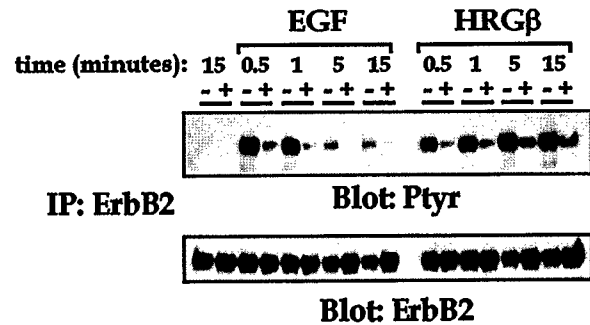


Figure 7. Tyrosine phosphorylation of erbB2 after EGF or HRG β stimulation is inhibited by the anti-erbB2 blocking Ab-16. MDA-MB-435 cells (5×10^6 per sample) were coated for 15 min on ice with either control mouse IgG (– lanes) or erbB2 Ab-16 (+ lanes) at $0.5 \mu\text{g}/1 \times 10^6$ cells. Cells were then stimulated for various times with 100 ng/ml EGF or HRG β at 37°C followed by lysis in an equal volume of $2\times$ lysis buffer. Immunoprecipitation for erbB2 was performed on cleared lysates, and samples were separated by SDS-PAGE. Immunoblotting for phosphotyrosine using 4G10 (upper panel) was performed after Western transfer. The membrane was then stripped and reprobed for erbB2 (lower panel) to confirm receptor levels. The figure shown is representative of a minimum of three independent assays.

phosphorylation and HRG β causing a rapid but more sustained phosphorylation of erbB2. Preincubation of cells with Ab-16 (+ lanes) dramatically reduced the activation of erbB2 phosphorylation by both EGF and HRG β , whereas incubation with control mouse IgG (– lanes) had no effect. Taken together, these data indicate that both EGF and HRG β stimulate their respective receptors, EGFR and erbB3, and subsequently cause increased phosphorylation of erbB2, presumably via a heterodimerization mechanism. However, although the anti-erbB2 Ab-16 functionally reduces the amount of tyrosine-phosphorylated erbB2 after stimulation with either growth factor, this antibody does not affect EGF-stimulated adhesion or migration (Figures 5 and 6), suggesting a differential role for erbB2 in EGF versus HRG β -mediated regulation of $\beta 1$ -integrin function.

Role of PI 3-K in EGF- and HRG β -induced up-regulation of $\beta 1$ -Integrin function

Because PI 3-K plays a role in signaling by the EGFR family members, we analyzed the contribution of this enzyme to the adhesion and migration events we had observed in the MDA-MB-435 cells. Stimulation of MDA-MB-435 cells with either EGF (Figure 8A) or HRG β (Figure 8B) resulted in rapid recruitment of the p85 subunit of PI 3-K to the phosphotyrosine-containing cellular fraction, consistent with previous reports of growth factor-stimulated PI 3-K activation in other cell lines (Carraway *et al.*, 1995). Analysis of *in vitro* PI 3-kinase activity from PY20 immunoprecipitates further demonstrated the increased activity of PI 3-K in response to EGF or HRG β stimulation of MDA-MB-435 cells (Figure 9). Consistent with the effects of EGF and HRG β on integrin-mediated adhesion, HRG β induced more potent PI 3-K activity than did EGF (Figure 9).

We tested the relevance of PI 3-K activation to adhesion with two pharmacologically distinct PI 3-K inhibitors, wort-

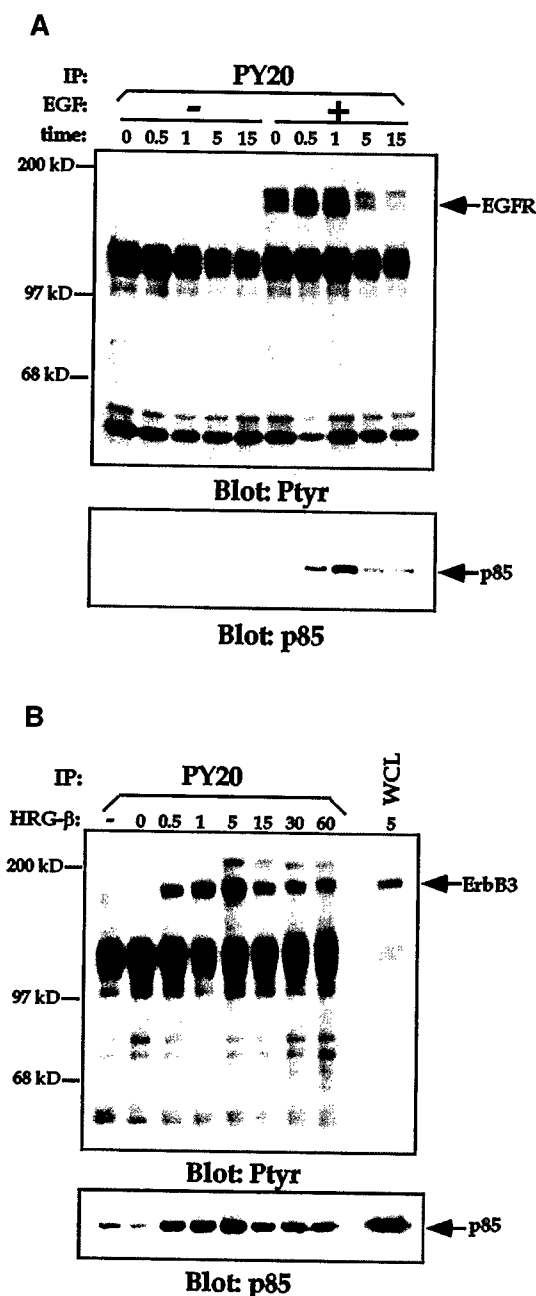


Figure 8. EGF and HRG β stimulation of MDA-MB-435 cells induces recruitment of the p85 subunit of PI 3-K to the phosphotyrosine cellular fraction. Cells were serum starved for 24 h and harvested as previously described. Cells (7.5×10^6 per sample) were incubated in the absence or presence of 100 ng/ml EGF (A) or 100 ng/ml HRG β (B) for the indicated periods at 37°C. Cells were then lysed, and phosphotyrosine-containing proteins were immunoprecipitated with the anti-phosphotyrosine mAb PY20 coupled to protein A-Sepharose beads. Washed immunocomplexes were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Western blotting was performed on membranes with the anti-phosphotyrosine mAb 4G10 to detect phosphotyrosine-containing proteins (upper panels). Blots were stripped and reprobed for EGFR or erbB3 (A and B, respectively; our unpublished data) and for p85 (lower panels). Similar data were obtained in at least two additional assays.

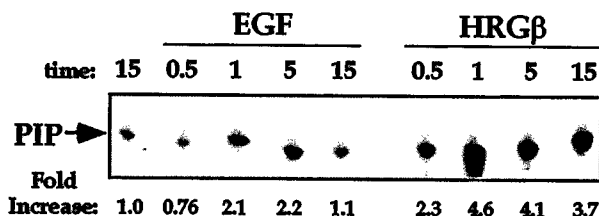


Figure 9. EGF and HRG β activate PI 3-K enzymatic activity in vitro. Cells were left unstimulated or stimulated in the presence of EGF or HRG β (each at 100 ng/ml) for various times at 37°C. PY20 immunoprecipitates were prepared for a PI 3-K in vitro kinase assay as previously described (Chan *et al.*, 1997). Kinase activity was detected by the in vitro phosphorylation of sonicated phosphatidylinositol, the products of which were visualized and quantitated after TLC separation, autoradiography, and phosphorimage analysis.

mannin and LY294002 (Arcaro and Wymann, 1993; Yano *et al.*, 1993; Okada *et al.*, 1994; Vlahos *et al.*, 1994). When cellular adhesion assays were performed in the presence of 100 nM wortmannin (Figure 10A), we observed a significant decrease in both EGF- and HRG β -stimulated MDA-MB-435 cell adhesion to COLL. In contrast, only small reductions in TS2/16-induced or unstimulated adhesion were observed. PI 3-K appears to contribute even more significantly to the process of migration in these cells, because wortmannin completely blocked HRG β -mediated migration on LAM (Figure 10B) as well as COLL (our unpublished data). Similar results were obtained when adhesion and migration were assessed in the presence of 25 μ M LY294002 (our unpublished data). In these experiments, unstimulated migration was also reduced, but only at the highest doses of the inhibitors. Although cells generally migrated in smaller numbers in experiments using EGF as a stimulus, motility induced by EGF on COLL and LAM was also strongly inhibited by wortmannin or LY294002 (Figure 10C).

As an alternative approach, we used a transient transfection assay that allowed us to assess the adhesion and migration of untransfected as well as transfected cells by flow cytometric analysis of adherent or migrated cell populations (Chan *et al.*, 1997; Kivens and Shimizu, 1999). Control vector expressing GFP alone or constructs expressing GFP-tagged wild-type p85 (GFP-wtp85) or a dominant negative p85 subunit (GFP- Δ p85) were transiently transfected into MDA-MB-435 cells followed by analysis in a modified cell adhesion assay. As shown in Figure 11, increasing levels of GFP alone had little effect on the adhesion of MDA-MB-435 cells under any stimulation condition, whereas expression of either GFP-wtp85 or GFP- Δ p85 subunits of PI 3-K decreased EGF or HRG β -mediated adhesion by ~50% without affecting TS2/16 or unstimulated adhesion significantly.

The effects of molecularly inhibiting PI 3-K function on HRG β - or EGF-stimulated cell migration of MDA-MB-435 cells were also investigated. Comparison of control transfected or GFP- Δ p85-transfected cell migration in standard Boyden chamber conditions revealed ~25% inhibition upon expression of GFP- Δ p85 in cells stimulated to migrate in the presence of HRG β , whereas no striking inhibition of unstimulated migration was apparent (Figure 12A). However, analysis of the specific GFP- Δ p85-positive cells in comparison with GFP-negative cells showed a striking inhibition of

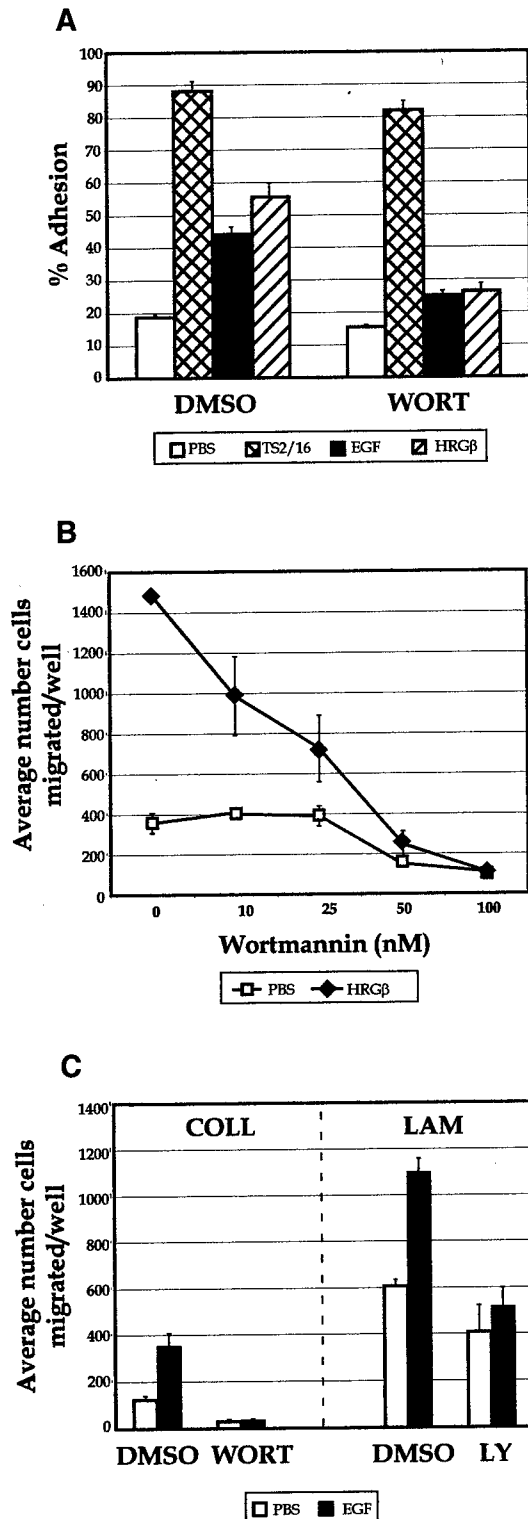


Figure 10. PI 3-K inhibitors block EGF- or HRG β -mediated adhesion and migration of MDA-MB-435 cells. (A) Adhesion assays were carried out in the presence of 100 nM wortmannin (WORT) with no stimulation (open bars) or after stimulation for 10 min at 37°C with TS2/16 (cross-hatched bars), EGF (shaded bars), or HRG β

both HRG β -stimulated and unstimulated cell migration with increasing expression of the GFP- Δ p85 construct (Figure 12B), in keeping with our results using wortmannin and LY294002. Similar results were obtained when cells were transfected with GFP-wtp85 or GFP- Δ p85 constructs and cell migration was stimulated with EGF (Figure 12C and D). Expression of the GFP protein alone had some inhibitory effect on overall migration, but the GFP-wtp85 or GFP- Δ p85-positive cells effectively blocked EGF-stimulated migration in comparison with levels of unstimulated cell motility. Thus, as demonstrated using both pharmacological and molecular inhibition methods, both EGF- and HRG β -stimulated pathways require functional PI 3-K for optimal $\beta 1$ -integrin-mediated adhesion and migration of MDA-MB-435 cells.

DISCUSSION

In this study we describe the activation of the $\beta 1$ -integrin in breast carcinoma cells upon EGF or HRG β growth factors binding to and activating members of the EGFR family of receptor tyrosine kinases. EGF treatment stimulated the rapid adhesion of MDA-MB-435 cells and increased cell migration toward ECM-coated surfaces in a $\beta 1$ -integrin-dependent manner. HRG β , a growth factor activating erbB3 in these cells, was even more potent at stimulating both adhesion and migration of breast carcinoma cells. Both EGF and HRG β use PI 3-K in pathways leading to increased adhesion and migration, and the more potent effects of HRG β on adhesion and migration are associated with a greater ability of HRG β to recruit and activate PI 3-K when compared with EGF. Furthermore, only HRG β -mediated signals were significantly affected by an antagonistic antibody toward erbB2, indicating a preferential contribution of heterodimers containing erbB2 and erbB3 rather than erbB2 and the EGFR in eliciting these effects on adhesion and motility.

Communication between Growth Factor Receptors and Integrins

We observed significant increases in EGF- and HRG β -mediated cell adhesion when binding was assessed on different ECM ligands, including FN, COLL, merosin, and LAM. Although the degree of HRG β -stimulated adhesion was consistently higher than that seen for EGF, direct activation of the $\beta 1$ -integrin with the monoclonal antibody TS2/16 was capable of stimulating cellular adhesion on all ECM components examined, suggesting that the complexity of the EGFR

Figure 10 (cont). (hatched bars). (B) Migration analysis was carried out in the presence of increasing amounts of wortmannin in the presence of media only (open squares) or HRG β (solid triangles). Similar effects were observed when dose-response analysis was carried out on COLL or when assays were performed in the presence of 25 μ M LY294002 (our unpublished data). (C) Migration on COLL or LAM was performed in the presence of control DMSO, 100 nM wortmannin (WORT), or 25 μ M LY294002 (LY) with no stimulation (open bars) or stimulation with 10 ng/ml EGF (solid bars). HRG β stimulation was slightly lower than normal in the adhesion assay shown in A in comparison with EGF or TS2/16 stimulation, but the data shown are otherwise representative of at least three separate experiments.

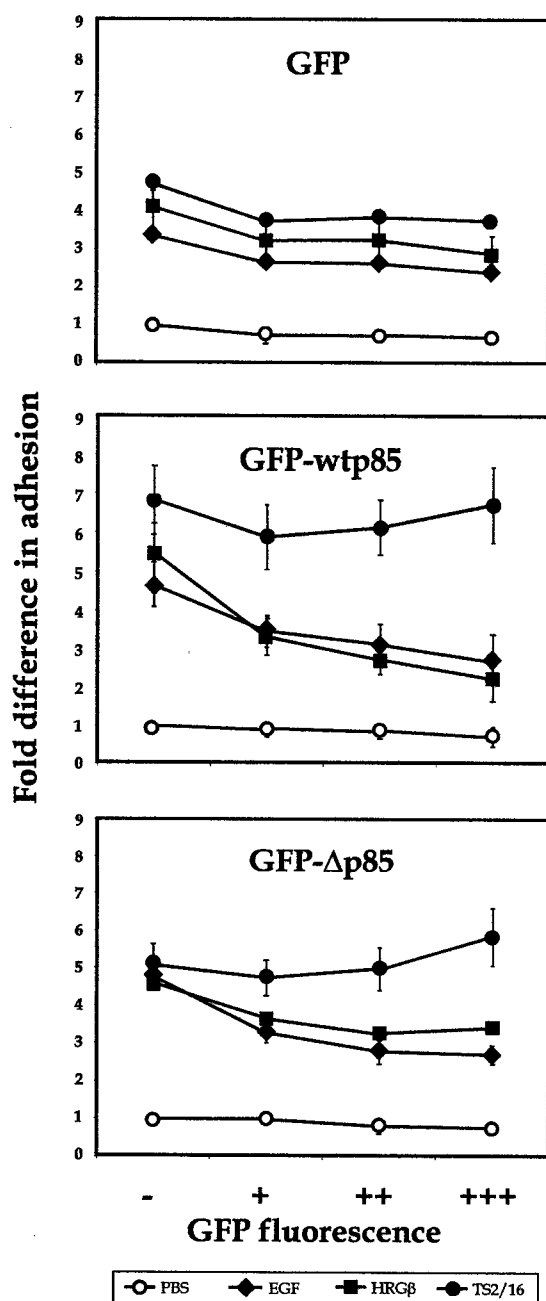


Figure 11. Overexpression of the wild-type or dominant negative p85 subunit of PI 3-K inhibits EGF- or HRG β -mediated increases in MDA-MB-435 adhesion to COLL. Control vector expressing GFP (top panel) or constructs expressing a GFP-wt p85 (middle panel) or GFP- Δ p85 (bottom panel) fusion protein were transiently transfected into MDA-MB-435 cells as described in MATERIALS AND METHODS. Transfected cells were allowed to recover for 24 h and then placed in serum-free media overnight. Cells were harvested as for standard adhesion assays, except that no Calcein AM labeling was performed, and cells (~300,000 cells per well) were added to 24-well plates coated with 6 μ g/well COLL. Adhesion was analyzed in the presence of PBS alone (open circles) or containing 1 μ g/well TS2/16 (solid circles), 100 ng/ml EGF (solid diamonds), or 100 ng/ml HRG β (solid squares) for 10 min at 37°C. Nonadherent cells were washed away, and adherent cells were collected from

family of receptors and their ligands plays a significant role in the response of these cells with respect to growth factor induction of integrin activation. Although other reports have described increases in cell adhesion upon overexpression of the EGFR (Lichtner *et al.*, 1993; Lichtner *et al.*, 1995; Verbeek *et al.*, 1998), the low levels of EGFR, erbB2, and erbB3 in the MDA-MB-435 cells are sufficient to result in growth factor-mediated changes in β 1-integrin-mediated adhesion and migration. Many reports have now illustrated the regulation of integrin function by cell surface receptors in a variety of cell types. Included in these are the increased adhesion through β 1-integrins initiated by the PDGF (Kinashi *et al.*, 1995) and c-kit (Kinashi and Springer, 1994; Serve *et al.*, 1995; Vosseller *et al.*, 1997) receptors. Although overexpression of the EGFR up-regulates adhesion of murine metastatic mammary cells (Lichtner *et al.*, 1993, 1995), high levels of the EGFR inhibited the function of RGD-sensitive integrin receptors in human squamous carcinoma cells (Fujii, 1996). In contrast to these previous studies with overexpressed EGFR, our studies demonstrate that modest levels of the EGFR can functionally activate the β 1-integrin in response to EGF or betacellulin stimulation in MDA-MB-435 breast tumor cells and further describe the potent increases in adhesion or migration initiated by HRG β binding to erbB3. Importantly, this up-regulation occurred in the absence of significant changes in integrin or EGFR family receptors on the cell surface (our unpublished data). Furthermore, our studies illustrate an additional level of complexity in EGFR family signaling by demonstrating differences in β 1-integrin activation through unique receptor pairs in response to EGF or HRG β .

Historically, experimental data have described signaling from growth factor receptors or from integrins independent of each other. However, a cell in its native environment likely experiences multiple stimuli, both from available growth factors and from integrin-mediated interactions with its physical surroundings. Several studies recently have been reported that describe the synergistic actions of integrin and growth factor receptor stimulation. For example, activation of the α v β 3-integrin and the PDGF receptor regulates endothelial cell migration (Woodard *et al.*, 1998) and potentiates PDGF-initiated signals (Schneller *et al.*, 1997), and insulin (Guilherme *et al.*, 1998) or interferon- γ (McCarthy *et al.*, 1997) receptor signals are potentiated by cell adhesion to β 1-integrin ECM ligands. Several recent reports have described cross-talk between the EGFR and integrins as assessed by activation of the MAP kinase pathway (Miyamoto *et al.*, 1996; Moro *et al.*, 1998; Wang *et al.*, 1998). EGF stimulation also induces tyrosine phosphorylation of the

Figure 11 (cont). wells using a 1:1 trypsin:1 mM EDTA solution. Collected cells were then analyzed by flow cytometry using aliquots of preadherent cell populations to confirm cell numbers added to wells for each transfectant and to determine the percent expression of GFP in the starting cell populations. Percent adhesion was determined by gating GFP-negative (-), GFP-low (+), -middle- (++) and -high (+++) positive cells and comparing preadherent and adherent cell numbers from each population. The data shown reflect fold differences in adhesion when compared with the GFP-negative, unstimulated cell subpopulation from each transfectant. Average percent adhesion was determined from samples examined in triplicate for each stimulation condition, and results were similar for a minimum of three independent assays.

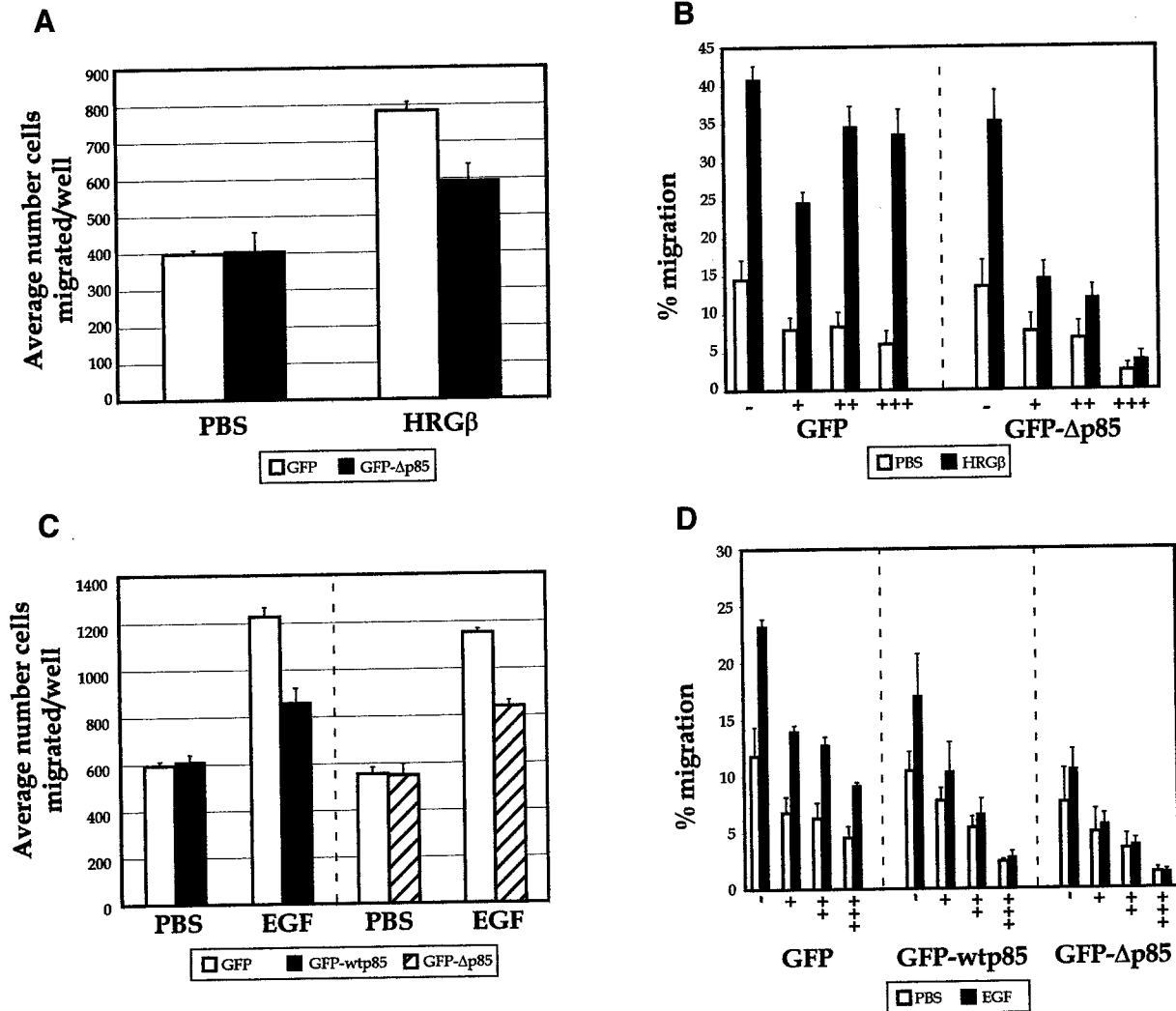


Figure 12. Overexpression of the dominant negative p85 subunit of PI 3-K inhibits HRG β - and EGF-mediated increases in MDA-MB-435 migration on LAM. Control vector expressing GFP or a construct expressing a GFP- Δ p85 fusion protein were transiently transfected into MDA-MB-435 cells. Transfected cells were allowed to recover for 24 h and then placed in serum-free media for 24 h. Cells were harvested and quantitated as previously described. (A) To monitor the efficiency of stimulated migration on bulk transfected populations, standard chemotaxis analysis was performed as described in MATERIALS AND METHODS with aliquots of cells at the same concentration as used for migration in transwells (below). Total cell populations expressing GFP (open bars) or GFP- Δ p85 (solid bars) were allowed to migrate in Boyden chambers in the presence or absence of HRG β in the lower wells on LAM-coated filters overnight, and migrated cells were fixed, stained, and quantitated as described. (B) The migration of GFP-positive versus GFP-negative cells in each bulk transfectant cells was assessed specifically by allowing cells to migrate overnight in transwell chambers that had been precoated with LAM in the absence (open bars) or presence of 100 ng/ml HRG β (solid bars). After ~16 h, migrated cells were collected from the lower surface of LAM-coated filters with trypsin/EDTA and analyzed by flow cytometry as described for transient adhesion assays in Figure 11. Data represent the average of three wells per condition and show the percent migration of each GFP-negative or GFP-positive cell population. (C) Cells expressing GFP (open bars), GFP-wtp85 (solid bars), or GFP- Δ p85 (hatched bars) were placed in the upper wells of Boyden chambers and allowed to migrate in the presence or absence of 10 ng/ml EGF on LAM-coated filters overnight, and migrated cells were fixed, stained, and quantitated as described. (D) The migration of GFP-positive transfected cells was assessed specifically by allowing cells to migrate overnight in transwell chambers that had been precoated with LAM in the absence (open bars) or presence of 10 ng/ml EGF (solid bars). After ~16 h, migrated cells were collected from the lower surface of LAM-coated filters with trypsin/EDTA and analyzed by flow cytometry as described for transient adhesion assays in Figure 11. Data represent the average of three wells per condition and show the percent migration of each GFP-negative or GFP-positive cell population. Migration observed under standard Boyden chamber conditions (A and C) reflected increases in migration in response to stimuli comparable with those seen in the transwell analysis. Modest effects of GFP-wtp85 or GFP- Δ p85 on migration were observed in the mixed cell populations in A and C but are more apparent upon single-cell analysis as shown in B and D. Comparison of assays carried out for 4 h vs. 16 h gave similar results, and the experimental data are representative of at least three independent assays.

$\alpha 6 \beta 4$ -integrin, suppresses its association with several signaling and cytoskeletal molecules, and increases $\alpha 6 \beta 4$ -dependent migration on LAM (Mainiero *et al.*, 1996). Clearly, a highly regulated and complex network of signaling is beginning to emerge that uses available integrins and growth factor receptor signaling complexes on a given cell type.

Regulation of EGF- and HRG β -stimulated Adhesion and Migration by PI 3-K

The identity of signaling molecules responsible for pathways of cellular communication between growth factor receptors and integrins has not been well characterized, but the requirement for PI 3-K activity in a variety of growth factor-regulated adhesion events has now been established, including those mediated by c-kit (Kinashi and Springer, 1994), PDGF (Kinashi *et al.*, 1995), and insulin (Guilherme *et al.*, 1998). Although evidence exists that PI 3-K is involved in unstimulated invasion by T47D (Keely *et al.*, 1997) and MDA-MB-435 breast carcinoma cells (Shaw *et al.*, 1997), our work has now demonstrated that ligand-activated EGFR family members also play an important role in augmenting $\beta 1$ -integrin-mediated adhesion and migration via activation of PI 3-K. Pharmacological inhibitors of PI 3-K gave strong inhibition of EGF- and HRG β -mediated increases in adhesion, and the overexpression of dominant negative forms of p85 supported this observation, as shown by an ~50% decrease in EGF- or HRG β -mediated adhesion with high levels of GFP- Δ p85. A significant decrease was also observed in the presence of high levels of GFP-wtp85, a result consistent with other studies demonstrating inhibition of downstream PI 3-K-dependent signaling upon overexpression of wild-type p85 (Rameh *et al.*, 1995; King *et al.*, 1997; Shaw *et al.*, 1997). In contrast to our adhesion data, both pharmacological and molecular inhibition of PI 3-K in our migration assays revealed PI 3-K as an essential component of EGF- or HRG β -driven signals to up-regulate $\beta 1$ -integrin-mediated migration, because treatment with wortmannin or LY294002 or overexpression of dominant negative p85 completely blocked EGF- or HRG β -induced cell migration.

Thus, our results suggest that growth factor receptors coupled to PI 3-K can play a critical role in tumor cell adhesion and motility by augmenting $\beta 1$ -integrin function. It is interesting to note that increased migration of MDA-MB-435 cells also occurs upon expression of a transfected $\beta 4$ -integrin subunit, which activates PI 3-K much more effectively than does the $\beta 1$ -integrin subunit (Shaw *et al.*, 1997). Thus, migration in untransfected MDA-MB-435 cells mediated by $\beta 1$ -integrins may require the activation of PI 3-K by growth factor receptors such as the EGFR family members. The cumulative activation of PI 3-K by a combination of growth factor receptors and integrins may therefore be critical to ensuring vigorous integrin-dependent migration of tumor cells. Our results are in contrast to a recent study that analyzed breast epithelial cell migration in response to EGF (Verbeek *et al.*, 1998). In this report, transfectants overexpressing the EGFR showed a dramatic increase in EGF-stimulated cell migration in comparison with parental ZR75-1 cells. Incubation with wortmannin and LY294002 enhanced EGF-mediated migration of EGFR-overexpressing cells, whereas the MEK1 inhibitor PD98059 decreased EGF-stimulated migration. However, no effect of either inhibitor was observed on parental cells, reported to express ~20,000

EGFRs on the cell surface compared with 1,200,000 EGFRs on transfected cells. The physiological relevance of effects of PI 3-K inhibitors on cells expressing such high levels of EGFR is currently unclear. Our results suggest that PI 3-K plays a positive role in both stimulated and unstimulated adhesion and migration of MDA-MB-435 cells, and we have noted little effect of inhibitors targeting the MAPK pathway (our unpublished data), consistent with reports by others (Shaw *et al.*, 1997). These results are also in keeping with recent data demonstrating the wortmannin-sensitive but PD98059-insensitive regulation of EGF-stimulated suppression of membrane ruffling and increased lamellipodia formation in rat nonmetastatic mammary adenocarcinoma cells (Wyckoff *et al.*, 1998), the HRG-inducible aggregation of breast cancer cells through a PI 3-K-dependent but MAPK-independent pathway (Tan *et al.*, 1999), and a role for PI 3-K in EGF-stimulated bladder cancer cell motility (Theodorescu *et al.*, 1998). These observations, and the fact that we see little effect of MAPK pathway inhibitors, are significant given the suggestion that subtle changes in levels and duration of MAPK activation might contribute to the differential outcome of specific growth factors acting on the same cell (Marshall, 1995; Pinkas-Kramarski *et al.*, 1998).

EGFR Family Heterodimerization and Regulation of $\beta 1$ -integrin Function

The importance of heterodimerization of EGFR family receptors for a variety of signaling processes is now clearly established (Earp *et al.*, 1995; Alroy and Yarden, 1997; Riese and Stern, 1998). For example, both qualitative and quantitative differences in EGFR receptor phosphorylation and recruitment of p85 have been described after activation with EGF versus HRG β in NIH3T3 cells expressing EGFR and erbB4 (Olayioye *et al.*, 1998). The emerging common theme is that erbB2 recruitment into a heterodimer with other members of the EGFR family greatly intensifies the signaling capacity initiated by a given growth factor binding to its cognate receptor (Graus-Porta *et al.*, 1995; Cohen *et al.*, 1996; Zhang *et al.*, 1996). Although the importance of the dimerization potential of this receptor family is now well accepted, virtually no work has been done to address the importance of these diverse signals in cellular adhesion and motility pathways. Our antibody blocking studies clearly show that the erbB2 receptor is a critical component of erbB3-mediated adhesion and migration pathways when stimulated by HRG β in MDA-MB-435 cells. This antibody reduces diverse aspects of both EGF and HRG signaling, including ligand-dependent tumor growth, DNA synthesis, and receptor dimerization (Klapper *et al.*, 1998). Our studies also confirm that this antibody reduces the transactivation effects of EGF and HRG β on erbB2 as illustrated by the dramatically diminished tyrosine phosphorylation of erbB2 after preincubation with Ab-16 (Figure 7). However, to our surprise, we observed no antagonistic effects of this antibody on EGF-mediated regulation of $\beta 1$ -integrin function, even at 10-fold higher than normal antibody:cell ratios (our unpublished observations), as measured by changes in cell adhesion or migration. This unexpected result reinforces the notion that pathways used for cell proliferation are likely overlapping but unique from those used in cell motility and adhesion, as previously suggested by others (Chen *et al.*, 1994). Furthermore, it suggests that, although the EGFR

requires erbB2 for optimal stimulation of cellular responses such as proliferation, erbB2 may not be a critical component of EGF-stimulated changes in β 1-integrin-dependent adhesion and migration on these cells.

It is intriguing that stimulation by HRG β is much more potent than EGF at mediating adhesion and migration of MDA-MB-435 cells, particularly given the kinase-impaired status of the erbB3 receptor. Although erbB2 tyrosine phosphorylation is increased in response to both growth factors (Figures 3 and 7), our antibody blocking data clearly suggest that erbB2 plays a prominent role in erbB3- but not EGFR-mediated regulation of the β 1-integrin. How this use of erbB2 differentially contributes to growth factor-mediated adhesion and migration is currently under investigation. Our data also reveal that both EGF and HRG β require functional PI 3-K for optimal adhesion and migration responses, although erbB3 is considered to be a more effective direct recruiter of the p85 subunit of PI 3-K, bearing multiple optimal p85 binding sites in its carboxyl-terminal tail (Hellyer *et al.*, 1998). It is possible that subtle differences in the mechanism of p85 activation and/or recruitment may contribute to the difference in strength of signals we observe between EGF and HRG β . Indeed, our immunoprecipitation analysis suggests a strong and more sustained recruitment of p85 to the phosphotyrosine cellular fraction upon HRG β stimulation compared with EGF (Figure 8). Furthermore, although p85 can be found in both erbB3 and erbB2 receptor immunoprecipitates after HRG β stimulation, we have not detected its coassociation with the EGFR after EGF activation in MDA-MB-435 cells (our unpublished observations). Other differences in recruitment of signaling molecules to or physical regulation of dimer complexes may also be involved. For example, the cbl molecule is primarily recruited to the EGFR and not to other members of the EGFR family (Levkowitz *et al.*, 1996), and the EGFR is significantly down-regulated from the cell surface in response to ligand binding, whereas erbB2, erbB3, and erbB4 are not regulated in the same manner (Baulida *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996). In further contrast, erbB3 does not bind to phospholipase C γ or GAP GTPase-activating protein (Fedi *et al.*, 1994), molecules commonly recruited to the ligand-activated EGFR. Given the current complexity of the EGFR family of receptors and their roles in the various cellular processes contributing to tumor cell formation and metastatic growth, it will be important to more clearly delineate the dimerization events undertaken by members of the EGFR family after the binding of specific ligands in a way that is informative for the signals generated by those ligands to up-regulate β 1-integrins. This complexity is exemplified by the fact that varying reports have described mitogenic, growth-inhibitory, or differentiative effects of HRG stimulation depending on the growth factor isotype, the concentration used, and the cell line studied (Graus-Porta *et al.*, 1995; Ram *et al.*, 1995; Hamburger and Yoo, 1997; Xu *et al.*, 1997). The consistent strength of response to HRG β stimulation in our studies suggests that this growth factor may play a significant role in the regulation of mammary tissue growth and development, as well as processes regulating cellular transformation in this tissue, through pathways communicating to β 1-integrins. Recent studies performed in a separate noninvasive breast cell line have also demonstrated the importance of PI 3-K as well as erbB2 recruitment in HRG-induced cytoskel-

etal reorganization and cell migration (Adam *et al.*, 1998). Thus, our current understanding of erbB3 and erbB4 signaling upon HRG binding suggests a prominent role in regulating both noninvasive and metastatic breast cells.

In summary, our data clearly demonstrate the ability of EGF-family growth factors to rapidly up-regulate β 1-integrin-mediated adhesion and enhance migration. We also have shown that EGF and betacellulin, ligands that activate the EGFR, and HRG β , a member of the neuregulin family of ligands that bind to and activate erbB3 and erbB4, induce dose- and time-dependent adhesion of MDA-MB-435 cells to COLL through mechanisms requiring functional PI 3-K. EGF and HRG β also up-regulate β 1-integrin-mediated migration on COLL and LAM, again using PI 3-K-dependent pathways. Studies with blocking antibodies further suggest a differential use of erbB2 by the erbB3 receptor rather than the EGFR upon growth factor binding. These results identify a novel functional outcome for stimulation by EGFR ligands and a critical role for EGFR signaling via PI 3-K in regulating integrin-dependent tumor cell adhesion and motility.

ACKNOWLEDGMENTS

We thank B. Vacchani for technical assistance, N.J. Maihle for critical reading of the manuscript, and S. Shaw, T. LeBien, and M. Kamps for providing antibody reagents. This work was supported by Department of Defense grant DAMD17-97-1-7228. Y.S. is the H. J. Kay Professor of Cancer Research at the University of Minnesota.

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